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Characterisation of microbial deep spoilage in Iberian dry-cured ham

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Abstract

The purpose of this work was to investigate the micro-organisms involved in overlooked "bone taint" spoilage of dry-cured Iberian hams. The physico-chemical characteristics of spoiled hams with 12 and 24 months of ripening, showing initial signs of alteration, were analyzed and their correlations with microbial counts studied. The spoilage potential of different microbial groups was assessed by the relationship between the microbial counts and the proteolysis level of spoilage as observed in the degradation of myofribrillar and sarcoplasmic protein fractions and in the changes in free amino acids. Non-enteric gram-negative bacteria (NEGN) were the dominant microbial group, showing a positive correlation with the moisture of spoiled hams. The Catalase-positive cocci (GPCP) growth was favoured by high NaCl concentrations in the spoiled hams, whereas the counts of *Enterobacteriaceae* were negatively affected by high NaCl concentration. The highest proteolytic microorganisms were the Gram-negative microbial groups playing *Enterobacteriaceae* a major role in the undesirable changes of the texture properties of the spoiled hams. With respect to the sensorial analysis, a synergy between NEGN and GPCP was observed in most of the strongly spoiled samples.

Keywords: Iberian dry-cured ham; Deep spoilage; Non-enteric Gram-negative bacteria; *Enterobacteriaceae*; Gram-positive Catalase-positive cocci

1. Introduction

Iberian dry-cured ham is a traditional meat product obtained after 24 months of ripening. During the first periods of processing, salting and slow drying are combined with a low temperature to reduce the risk of bacterial spoilage. In the final product, the pH values typically range from 5.6 to 6.5, and salt content reaches values of around 6–10% NaCl, bringing a_w values down to 0.85 or even lower (Rodríguez et al., 1994).

The most important cause of spoilage in Iberian and other kinds of dry-cured ham is called "bone taint" or "deep spoilage". This alteration occurs most commonly in the large muscle masses adjacent to the bone structures, and is characterized by a pasty texture and foul-smelling odour. The texture properties of Iberian dry-cured ham are considered to be one of the most important attributes affected in "deep spoilage" as a consequence of the major degradation of the structural components of muscle. The microbial growth which is the cause of this proteolytic activity results in a pasty texture and in the generation of peptides, amino acids, amines, ammonia, sulfides, alcohols, aldehydes, ketones, and organic acids with unpleasant and unacceptable off-flavours (García, Martín, Timón, & Córdoba, 2000). The off-odours and flavours that develop during the ripening process have previously been described as slightly acid to putrid (Córdoba, Aranda, & Benito, 2001). Deep spoilage often means the loss of the entire ham, but it could be slight and overlooked in the piece, remaining undetected until consumption.

The prevalent microbial groups in fully spoiled Iberian dry-cured hams are *Enterobacteriaceae* and Gram-positive

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Catalase-positive cocci, but lactic acid bacteria (LAB) have also been reported at low levels (García et al., 2000). Several studies have considered the *Enterobacteriaceae* group to be the bacterial population responsible for spoilage of different kind of dry-cured hams, including Iberian dry-cured ham (Marín, Carrascosa, & Cornejo, 1996; Miranda, Ordóñez, Jaime, & Rovira, 1998; Paarup, Nieto, Peláez, & Reguera, 1999; García et al., 2000). Species of the genera *Serratia*, *Enterobacter*, *Proteus*, *Leclercia*, and *Hafnia* have been the most frequently isolated from spoiled hams. It would be of great interest to know the microbial population of deep slight and overlooked spoilage in the piece that usually happens at an advanced stage in the ripening process. In this case, local conditions of humidity and NaCl concentration could favour microbial growth.

On the other hand, the effects of the microbial groups reported to be responsible for deep spoilage remain unclear. To control this microbial alteration, one needs to know how the different groups affect the level of proteolysis and sensory characteristics of Iberian dry-cured hams.

The present work is a study of the microbial population of dry-cured ham with signs of deep spoilage overlooked in the pieces. In addition, the level of hydrolysis of myofibrillar and sarcoplasmic proteins, changes in free amino acids, and sensory properties were determined and correlated with the microbial population.

2. Materials and methods

2.1. Sample collection

The study included 30 spoiled Iberian hams, taken from the ripening process at 12 and 24 months when they showed initial sings of spoilage (off-odour). In all of the spoiled hams the alteration was overlooked in the internal muscle (*biceps femoris*). The off-odour of these hams was detected after internal puncture with a thin bone. Three hams without internal off-odour were sampled as controls for each stage, 12 and 24 months of ripening.

Samples (15 g approx.) were aseptically collected with a 2.5-cm diameter sterile metal cork borer from approximately the geometric centre of the ham, near to the coxofemoral joint (Martín, Córdoba, Núñez, Benito, & Asensio, 2004). The inner most part of the cylinder consisting of the *biceps femoris* as the basis muscle was selected for microbial analysis. The adjacent zone to this muscle (50 g approx.) was taken for the physico-chemical and sensory analyses.

2.2. Microbiological analysis

In order to carry out the counts and isolates, 10 g of the internal sample of ham were homogenized in 90 ml sterile 0.1% peptone in a Stomacher (Lab Blender, Model 4001, Seward Medical, London, UK) for 30 s. Appropriate dilutions were made with 0.1% peptone broth and 1 ml were plated onto the culture media under the following conditions. Total counts on Plate Count Agar (PCA, Oxoid, Unipath, Basingstoke, UK) for 72 h at 30 °C; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA, Oxoid) for 24 h at 37 °C; lactic acid bacteria, LAB, on MRS Agar (Oxoid) in anaerobic conditions for 72 h at 30 °C; Gram-positive Catalase-positive cocci on Mannitol Salt Agar (MSA, Oxoid) after 72 h at 37 °C; sulfite reducing clostridia on Sulfite–Polymyxin–Sulfadiazine (SPS) agar incubated anaerobically for 72 h at 37 °C; intestinal enterococci on Slanetz and Bartley agar (S&B, Oxoid) for 24 h at 37 °C; yeasts and moulds on Malt Extract Agar (MEA, Oxoid) for 4 days at 25 °C.

For the samples with counts higher than 2 log cfu g^{-1}, the colonies were selected according to morphology from plates that showed counts between 30 and 300 colonies (2–5 colonies for each plate), and were then subcultured on the same medium on which they had been isolated. Each isolate was examined for colony and cellular morphology under a microscope, and in the case of bacteria was tested for their Gram reaction. Complementarily, catalase, oxidase, and urease activities, and glucose and lactose fermentation, were tested to characterize the colonies at the microbial group level.

The different microbial group strains were tentatively identified by biochemical profile. Lactic acid bacteria (LAB) were characterized by the API 50 CH and Gram-positive Catalase-positive cocci (GPCP) by the API-Staph systems (both from BioMérieux, Geneva, Switzerland), and *Enterobacteriaceae* by API 20E and non-enteric Gram-negative bacteria (NEGN) by API 20 NE (BioMérieux). The identification of the strains with different biochemical profiles was confirmed by the sequence analysis of the gene encoding 16S rDNA.

2.3. 16S rDNA sequence analysis

16S rDNA gene PCR amplifications of extracted DNA (5 ml) were performed as described previously (Aranda, Rodríguez, Asensio, & Córdoba, 1997) with two different primers: Primers pA (5′-AGAGTTTGATCCTGGCTCAG; nucleotides 8 to 28 of the 16S rDNA gene of *Escherichia coli*) and reverse oligonucleotide R609 (5′-TTTCACGAACAACGCGACAA; nucleotide positions 609 to 590 of the *E. coli* 16S rDNA gene) for PCR amplification of a 590-bp sequence of the 16S rDNA gene including the V2 and V3 regions (Hughes et al., 2000). Ten microlitres of extracted DNA were added as template in 50 μl reaction mixtures containing 50 pmol of primers, 500 mM of each dNTP, 0.1 vol of 10X PCR buffer (500 mM 23 Tris–HCl, pH 9.2, 140 mM (NH4)2SO4, 22.5 mM MgCl2), and 1 U Taq polymerase (Biotools B&M Labs, S.A., Madrid, Spain). Amplification mixtures were subjected to 4 min of denaturation at 94 °C; 30 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; followed by a final extension period of 7 min at 72 °C and refrigeration in a model-480 thermal cycler (Perkin–Elmer Corporation, Norwalk, Conn.). Sequence analysis of the purified PCR products was performed at the Sequence

Centre, SECUGEN S.L., Madrid, Spain, with primers pA and R609. Sequences were edited with Chromas version 1.43 (Griffith University, Brisbane, Queensland, Australia), and sequence comparisons were made with EMBL and GenBank database sequences.

2.4. Physico-chemical parameters

The moisture content of the hams was determined after dehydration at 100 °C to constant weight following ISO-1442 method. The pH was measured using a Crison model 2002 pH meter (Crison Instruments, Barcelona, Spain). Chloride, nitrate, acetate, and lactate anions were assayed by capillary zone electrophoresis (CZE) using an automated PACE 5500 (Beckman Instrument, Inc. Palo Alto, CA, USA) equipped with a 50 μm diameter, 27 cm total length (20 cm until window detector), uncoated fused silica capillary from Supelco (Tecknocroma, Barcelona, Spain). The method described by O'Flaherty, Yang, Sengupta, and Cholli (2001) for inorganic and organic anions analysis was followed. Before assay, the homogenate of the sample with distilled water (1:50 w/v) was filtered through a 0.2 μm filter.

2.5. Sarcoplasmic and myofibrillar proteins analysis

To know the level of hydrolysis of sarcoplasmic and myofibrillar proteins, these protein fractions were analyzed in spoiled hams. To obtain the sarcoplasmic protein extract, 2 g of sample was homogenized with 20 ml of 0.03 M, pH 7.4 sodium phosphate buffer (Benito, Rodríguez, Sosa, Martín, & Córdoba, 2003) in a Sorvall omnimixer (Omni Corporation International Instruments, Waterbury, CT, USA). The extract was centrifuged at 8000*g* for 15 min at 4 °C, and the supernatant was filtered through a 0.45 μm filter. The myofibrillar protein extract was obtained after extraction of the resulting pellet with 20 ml of 1.1 M potassium iodide + 0.1 M sodium phosphate, pH 7.4 buffer, following the procedure described above.

The sarcoplasmic and myofibrillar protein hydrolysis was analyzed by SDS–PAGE. The protein extracts were electrophoresed on a 7.5% (wt/vol) SDS–PAGE following the standard method (Laemmli, 1970), loading the wells of the electrophoresis gel with 4 μl of sample denatured by boiling for 5 min in 0.0625 M Tris–HCl buffer at pH 6.8 with 20% glycerol, 2% SDS, and 5% 2 β-mercaptoethanol. Proteins were visualised by Coomassie Brilliant Blue R-250 staining. Molecular Weight Marker from 205 kDa to 14.5 kDa (Sigma Chemical, St. Louis, MO, USA) was used as standard. The density of the protein bands was measured using the Kodak Digital Science software package (Kodak Digital Science, Rochester, New York, USA).

2.6. Free amino acid analysis

For deproteinization, 10 g of sample was mixed with 20 ml of 10% sulfosalicylic acid containing norleucine (0.5 mg/ml) as internal standard. Then, amino acids were determined following the procedure described by Córdoba et al. (1994b) on a Beckman liquid chromatograph (Beckman Instruments, Inc. Palo Alto, CA, USA) equipped with two pumps (Model 126) and a UV detector (Model 166). The column was a Luna containing octadecyldimethylsilyl, 25 cm × 4.6 mm (5 μm particle size) from Phenomenex (Torrance, CA, USA).

2.7. Sensorial analysis

Eighteen panelists trained with different samples of spoiled and unspoiled hams were asked to characterize spoilage level. The only sensory descriptor used was "spoilage level". During each session, six spoiled hams were presented in randomized order to the panelists who judged the spoilage level ordering the samples from minor to major spoilage using a numbered scale (from 1 to 6 points). Each sample was evaluated twice and classified according to average value in different spoilage levels (LS: lightly spoiled between 0 and 2 points; MS: moderately spoiled between 2 and 4 points; SS: strongly spoiled between 4 and 6 points).

2.8. Statistical methods

Statistical analysis of the data were carried out using SPSS for Windows, 10.0. (SPSS Inc., Chicago, IL, USA). Mean, median, standard deviation, minimum, and maximum values were calculated for the physico-chemical parameters, and the effects of these factors on microbial counts in the spoiled hams were evaluated and tested using multiple linear regression. A principal component analysis (PCA) was performed on the protein and microbial count data to study the effect of the different microorganisms on the degradation of the myofibrillar and sarcoplasmic fractions. The relationships between microbial counts and amino acid values were evaluated by Pearson correlation coefficients, and verified by linear regression analysis. With respect to the sensory data, these were represented as a matrix of 30 rows (spoiled Iberian dry-cured hams) and 5 columns (4 count values of microbial groups plus the sensory descriptor), and a PCA was used to construct the linear combination with maximal variance.

3. Results and discussion

3.1. Counts of the different microbial groups

Counts of the microorganisms in spoiled and unspoiled hams are presented in Table 1. PCA counts showed higher levels than those of the remaining culture media in both batches of spoiled ham (12 and 24 months of ripening). All the spoiled hams with 12 months of ripening had counts greater than to 2 log cfu g^{-1}, with 50% exceeding 7 log cfu g^{-1}. PCA counts in the spoiled hams with 24 months of ripening had in general declined, probably as a consequence of the reduction in moisture and increased

Table 1
Microbial counts of the Iberian dry-cured hams studied

Samples	Microbial counts (log cfu g^{-1})						
	SPS	VRBG	S&B	MRS	MSA	MEA	PCA
Hams with 12 months ripening							
Control ham 1	<2[a]	<2	<2	<2	<2	<2	2.6
Control ham 2	<2	<2	<2	<2	<2	<2	2.0
Control ham 3	<2	<2	<2	<2	<2	<2	2.6
Spoiled ham 1	<2	<2	<2	<2	3.2	<2	8.2
Spoiled ham 2	<2	3.0	<2	<2	<2	<2	8.3
Spoiled ham 3	<2	2.2	<2	<2	<2	<2	8.0
Spoiled ham 4	<2	<2	<2	4.0	2	<2	8.0
Spoiled ham 5	<2	2.1	<2	<2	<2	<2	8.2
Spoiled ham 6	<2	6.0	<2	<2	2.0	<2	6.0
Spoiled ham 7	<2	<2	<2	<2	3.3	<2	6.2
Spoiled ham 8	<2	<2	<2	<2	2.3	<2	8.0
Spoiled ham 9	<2	2.0	<2	<2	<2	<2	7.1
Spoiled ham 10	<2	<2	<2	<2	<2	<2	7.5
Spoiled ham 11	<2	<2	<2	4.0	2.0	<2	4.6
Spoiled ham 12	<2	<2	<2	4.9	<2	<2	4.3
Spoiled ham 13	<2	<2	<2	<2	2.3	<2	4.5
Spoiled ham 14	<2	<2	<2	3.3	3.4	<2	3.1
Spoiled ham 15	<2	<2	<2	<2	2.5	<2	5.7
Hams with 24 months ripening							
Control ham 4	<2	<2	<2	<2	2.1	<2	<2
Control ham 5	<2	<2	<2	<2	<2	<2	<2
Control ham 6	<2	<2	<2	<2	2.5	<2	<2
Spoiled ham 16	<2	2.1	<2	<2	<2	<2	4.5
Spoiled ham 17	<2	<2	<2	<2	2.0	<2	4.3
Spoiled ham 18	<2	<2	<2	4.1	<2	<2	<2
Spoiled ham 19	<2	<2	<2	2.3	2.0	<2	<2
Spoiled ham 20	<2	<2	<2	2.1	<2	<2	<2
Spoiled ham 21	<2	<2	<2	3.5	<2	<2	3.5
Spoiled ham 22	<2	3.5	<2	<2	<2	<2	3.2
Spoiled ham 23	<2	<2	<2	<2	<2	<2	4.9
Spoiled ham 24	<2	2.2	<2	<2	<2	<2	3.0
Spoiled ham 25	<2	<2	<2	<2	6.0	<2	6.0
Spoiled ham 26	<2	<2	<2	<2	5.0	<2	3.7
Spoiled ham 27	<2	<2	<2	<2	2.5	<2	4.7
Spoiled ham 28	<2	<2	<2	<2	4.0	<2	4.8
Spoiled ham 29	<2	<2	<2	<2	2.7	<2	2.3
Spoiled ham 30	<2	<2	<2	<2	2.0	<2	4.0

[a] <2: counts lower than 2 log cfu g^{-1}.

NaCl concentration. However, the counts were still greater than 4 log cfu g^{-1} in almost 50% of the hams. In the control batches, the PCA counts were always less than 3 log cfu g^{-1}. Most of strains isolated from the culture media PCA were Non-enteric Gram-negative bacteria (NEGN) according to the cellular morphology, catalase and oxidase activities. This microbial group has not been described as causing spoilage of dry-cured ham, although counts greater than 6 log cfu g^{-1} of unidentified total aerobic populations have been reported in spoiled "Serrano" and Iberian dry-cured hams (Miranda et al., 1998; García et al., 2000).

MSA counts were less than 4 log g^{-1} in both batches of spoiled hams and in the control batches (Table 1). Gram-positive Catalase-positive cocci (GPCP) were the only microbial group isolated from MSA agar. These microorganisms have been identified as predominant during most of the ripening time in the normal processing of different types of dry-cured hams (Huerta, Hernández, Guamis, & Hernández, 1988; Rodríguez et al., 1994; Losantos, Sanabria, Cornejo, & Carrascosa, 2000). In Iberian ham, GPCP counts of 4 log g^{-1} have been found in deep tissues at the end of the post-salting period (Rodríguez et al., 1994) and have not been associated with spoilage of dry-cured ham (García et al., 2000). This microbial group has been reported to contribute to colour and flavour development in dry-cured meat products (Lücke, 1986).

Regarding VRBGA counts only 33% and 20% of the spoiled hams with 12 and 24 months ripening, respectively, had counts greater than 2 log cfu g^{-1}, while in the control batch this microbial group was not detected (<2 log cfu g^{-1}). *Enterobacteriaceae* were the only strains isolated in this culture medium. This microbial group has been reported at levels of 2–6 log cfu g^{-1} in spoiled Iberian dry-cured hams (García et al., 2000; Losantos et al., 2000), so that these microorganisms are attributed a major role in spoilage.

MRS Agar showed counts greater than 2 log cfu g^{-1} in 27% of the hams of both spoiled batches, but always with counts below 5 log cfu g^{-1} (Table 1). Most of the strains isolated in this culture medium were Lactic acid bacteria (LAB). LAB counts of around 2 log cfu g^{-1} have been found in spoiled and unspoiled Iberian dry-cured ham (García et al., 2000). Finally, counts in SPS and S&B culture media were irrelevant, with levels below 2 log cfu g^{-1} in both the spoiled and unspoiled control batches. Microorganisms growing in these culture media were therefore discarded from consideration for the characterization and identification of the isolates.

3.2. Identification of microbial isolates

From a total of 129 strains isolated, 60 were identified as NEGN, 28 as GPCP, 25 as *Enterobacteriaceae*, and 16 as LAB (Table 2). No differences were found between biochemical and 16S rDNA sequence analysis for most of the isolates, except for the LAB. Most of LAB strains were identified as *Lactobacillus delbrueckii* by biochemical profile, whereas these isolates were identified as *Lactobacillus salivarus* and *Lactobacillus curvatus* by 16S rDNA sequence analysis. In addition, several strains identified at genus level by biochemical profiles were finally identified at species level by 16S rDNA sequence analysis.

The same species of the NEGN population were isolated from the two batches of spoiled ham. The most frequent NEGN isolates were *Aeromonas hydrophila* (23.8% frequency of total isolates) followed by *Burkholderia cepacea* (11.1%) and *Vibrio fluvialis* (7.9%) (Table 2). *Agrobacter radiobacter* were also isolated but at lower percentages (4.8%). *Aeromonas hydrophila* strains were isolated from 60% and 53.3% of the spoiled hams with 12 and 24 months of ripening, respectively, with counts that reached 8 log g^{-1} in the batch with 12 months of ripening. Similar counts were obtained for *Burkholderia cepacea* and *Vibrio fluvialis*, but their prevalence was less than 20% in both batches of

Table 2
Microbial prevalence and counts in the Iberian dry-cured ham batches studied

Microorganisms	Strains		Spoiled hams				Unspoiled hams			
			12 months		24 months		12 months		24 months	
	No.	%	%[a]	log cfu g^{-1}	%	log cfu g^{-1}	%	log sfu g^{-1}	%	log sfu g^{-1}
NEGN	60	47.6	100		80		66.7		0	
Aeromonas hydrophila	30	23.8	60	4.3–8.3	53.3	3.5–4.5	33.3	2.6	0	<2
Burkholderia cepacea	14	11.1	13.3	3.4–8.2	13.3	3–4.8	33.3	2.6	0	<2
Vibrio fluvialis	10	7.9	20	5.7–8.3	6.7	5.7	0	<2	0	<2
Agrobacter radiobacter	6	4.8	6.7	7.5	6.7	2.0	0	<2	0	<2
GPCP	28	22.2	60		53.3		33.3		66.7	
Staphylococcus lentus	14	11.1	46.7	2.0–3.3	20	2.7–5.0	0	<2	0	<2
Staphylococcus xylosus	14	11.1	13.3	2.3–3.2	33.3	2.0–6.0	33.3	2.0	66.7	2.1–2.5
Enterobacteriaceae	22	17.5	33.3		20		0		0	
Serratia liquefaciens	10	7.9	13.3	2.0–3.0	13.3	2.0	0	<2	0	<2
Serratia odorifera	9	4.8	13.3	2.0–6.0	0	<2	0	<2	0	<2
Hafnia alvei	4	3.2	6.7	2.0	6.7	3.5–2.0	0	<2	0	<2
Enterobacter aerogenes	2	1.6	6.7	2.0	0	0	0	<2	0	<2
LAB	16	12.7	26.7		26.7		0			
Lactobacillus salivarius	14	11.1	20	3.0–4.0	26.7	2.0–4.0	0	<2	0	<2
Lactobacillus curvatus	2	1.6	6.7	4.9	0	<2	0	<2	0	<2
Total	126	100								

[a] Incidence per sample of species identified.

spoiled hams (Table 2). *Aeromonas* spp. and *Vibrio* spp. have been reported as habitual microorganisms in fresh meat, cooked ham, and smoked sausages, but always with counts lower than those found in the present study (Gobat & Jemmi, 1995; Encinas, Gonzalez, García-López, & Otero, 1999; Villari, Crispino, Montuori, & Stanzione, 2000; Devlieghere, Lefevere, Magnin, & Debevere, 2000; Daskalov, 2006). Although these microorganisms have not previously been described in the microbial population of spoiled dry-cured ham, both genera have been isolated as spoilage bacteria in lightly preserved products such as cold-smoked salmon and different kinds of cured meats (Gardner, 1980; Hinrichsen, Montel, & Talon, 1994; Joffraud, Leroi, Roy, & Berdague, 2001). *Aeromonas* spp. and *Vibrio* spp. have been found normally in the processing equipment of pig slaughtering plants (Gill & Jones, 1995; Gill, Badoni, & McGinnis, 1999), indicating that their presence in raw hams results naturally from the carcass-dressing process.

With respect to the GPCP, 28 strains (22.2% frequency of total isolates) were isolated and identified, all corresponding to the genus *Staphylococcus* (Table 2). We isolated *S. xylosus* from all four batches of hams, but *S. lentus* only from the spoiled ham batches. Strains of *S. xylosus* were mainly isolated in spoiled ham with 24 months of ripening and in the control batches. This microorganism has commonly been isolated from different types of dry-cured ham (Rodríguez, Nuñez, Córdoba, Bernúdez, & Asensio, 1996; Carrascosa & Cornejo, 1991; Molina, Silla, Flores, & Mozo, 1990), and has been reported to be involved in the flavour development of cured meat products (Stanhke, 1994). The predominant *Staphylococcus* species in spoiled hams, especially in the batch with 12 months of ripening, was *S. lentus*. Strains of this microorganism have been isolated from other cured meats, such as sausages (Coppola, Mauriello, Aponte, Moschetti, & Villani, 2000), and their origin could be associated with contamination f rom the skin and faeces of the pigs during the processing of the raw ham (De Boer, Slaughter, Applegate, Sobieski, & Crupper, 2001).

Serratia proteamaculans and *Serratia odorifera* constituted most of the *Enterobacteriaceae* strains isolated in the spoiled ham batches (Table 2). Isolates of *Hafnia alvei* (3.2%) and *Enterobacter aerogenes* (1.6%) were also obtained in both spoiled ham batches. The genera *Serratia*, *Hafnia*, and *Enterobacter* have been indicated as the main cause of deep spoilage in different types of hams (Miranda et al., 1998; Paarup et al., 1999; García et al., 2000).

The most abundant LAB species was *Lactobacillus salivarius* with a total of 14 strains (11.1% of total isolates) and a prevalence of 20% and 26.7% in the spoiled hams with 12 and 24 months of ripening, respectively. Only 2 strains, isolated from the batch of spoiled ham with 12 months of ripening were identified as *Lactobacillus curvatus*. These two species have not previously been described in dry-cured ham, but they have been reported as usual in other meat products under similar pH and NaCl conditions, such as in dry-cured meat sausages (Sanz et al., 1999; Rantsiou & Cocolin, 2006).

3.3. Relationship between physico-chemical parameters and microbial counts in spoiled hams

Table 3 shows the physico-chemical characteristics of the spoiled Iberian hams analyzed and their correlations with the microbial counts. The mean moisture content

Table 3
Physico-chemical parameters of spoiled Iberian dry-cured hams and correlation with microbial counts

Physico-chemical parameters	Spoiled hams			Correlations with microbial counts[a]			
	12 months	24 months	P[b]	NEGN	GPCP	Ent.	LAB
Moisture (%)	48.9; 49.5 (40.7–55.4)[c]	37.6; 39.5 (26.4–47.7)	0.001	0.551**	0.202	0.118	0.012
NaCl (%)	5.3; 5.3 (1.5–9.1)	6.8; 6.8 (4.1–9.6)	0.115	−0.161	0.285*	−0.247*	0.111
pH	6.6; 6.6 (6.3–7.0)	6.7; 6.6 (6.1–7.6)	0.575	0.045	0.475**	−0.062	0.064
Lactate (%)	0.380; 0.398 (0.058–0.637)	0.369; 0.323 (0.146–0.653)	0.869	−0.090	0.023	0.178	0.191
Acetate (%)	0.085; 0.025 (0.007–0.707)	0.108; 0.071 (0.028–0.489)	0.720	−0.099	0.055	0.515**	−0.105

[a] Ent: *Enterobacteriaceae*; NEGN: Non-enteric Gram-negative bacteria; GPCP: Gram-positive catalase-positive cocci. Regression coefficients of main effect.
[b] P: P-value due to time of ripening sampled.
[c] Mean, median, minimum, and maximum of physico-chemical parameters in the two batches of spoiled Iberian dry-cured hams.
* $P < 0.05$.
** $P < 0.001$.

was 48.9% and 37.6% for the hams with 12 and 24 months of ripening, respectively. Similar values of moisture have commonly been reported for both normally processed and spoiled Iberian dry-cured hams (Blanco et al., 1997; García et al., 2000). As expected, the moisture and NEGN counts were positively correlated but the growth of this microbial group was not relevantly affected by the NaCl concentrations found in the spoiled hams (an average of 5.3 and 6.8% (w/w) for the hams with 12 and 24 months of ripening, respectively) with a correlation coefficient of 0.181. Indeed, the halotolerant Gram-negative bacteria *Aeromonas* spp. and *Vibrio* spp. have been reported as the dominant microorganisms involved in the spoilage of salted food such as cod (Rodrígues, Ho, Lopez-Caballero, Vaz-Pires, & Nunes, 2003). The growth of GPCP was favoured by high NaCl concentrations in the hams, showing this microbial group to be well-adaptated to the ecological conditions of the Iberian dry-cured ham. On the contrary, the *Enterobacteriaceae* counts declined with increasing NaCl concentration. Indeed, the presence of *Enterobacteriaceae* due to insufficient NaCl concentration during curing has been described as the cause of deep spoilage in White dry-cured hams (Paarup et al., 1999).

With respect to pH, the high values of both spoiled batches are consistent with other reports (Miranda et al., 1998; García et al., 2000). The increase in pH seemed mainly to be favoured by the increase of GPCP counts, since these microorganisms have often been reported to be amine producers from amino acid decarboxylation in meat products (Silla Santos, 1998; Martuscelli, Crudele, Gardini, & Suzzi, 2000). The *Enterobacteriaceae* counts correlated positively with increases in acetic acid. This finding is consistent with data in the literature indicating that acetic acid and other organic acids are produced by *Enterobacteriaceae* on cured meat products (García et al., 2000).

3.4. Protein degradation potential of the microbial population

A total of 23 bands ranging from 9 to 400 kDa, were detected in the myofibrillar fraction of spoiled hams with 12 and 24 moths of ripening. Five of these were tentatively identified by their molecular weights as tropomyosin (34 kDa), actin (46 kDa), α-actinin (88 kDa), C protein (122 kDa), and H-meromyosin (193 kDa). However, the 46 kDa band could include other peptides such as myosin chain fragments (Benito et al., 2003; Luccia et al., 2005). The 55 kDa band may include desmin, vimentin, and Z protein (Maruyama, 1985), and the rest of the peptides consist of proteolytic products from proteins of higher molecular weight. The hydrolysis of myofibrillar proteins plays a major role in the texture properties of Iberian dry-cured ham. When the extent of proteolysis is excessive, the structure is severely damaged and unpleasant textures appear (Todrá et al., 2006).

There were major differences in the myofibrillar protein profiles of the spoiled ham samples. The PCA of the intensity of the proteins and peptides of the myofibrillar fraction and the microbial counts obtained for each spoiled ham is illustrated in Fig. 1. The first axis accounts for 28.6% of the variance, and was mainly defined by most of the myofibrillar proteins and peptides probably deriving from their degradation. The results showed the relationship between the *Enterobacteriaceae* counts and the degradation of these myofibrillar proteins. In particular, the counts of this microbial group had a strong negative correlation (at the 95% confidence level) with troponin and tropomyosin, and a positive correlation with the peptides of 20 and 23 kDa. The high proteolytic activity of *Enterobacteriaceae* isolated from spoiled hams has been reported in previous work (Losantos et al., 2000). We observed no major relationship however between the degradation of myofibrillar proteins and the counts of the other microbial groups (NEGN, GPCP, and LAB), showing that these microorganisms have less influence on the texture properties of the spoiled hams that the *Enterobacteriaceae*.

On the second axis (21.2% of the variance), myosin is located at the top of the figure whereas peptides of 58 and 66 kDa, probably products of myosin hydrolysis, are at the bottom. The microbial groups did not appear to be the main cause of myosin degradation, which could be associated with the difference of endogenous enzymatic

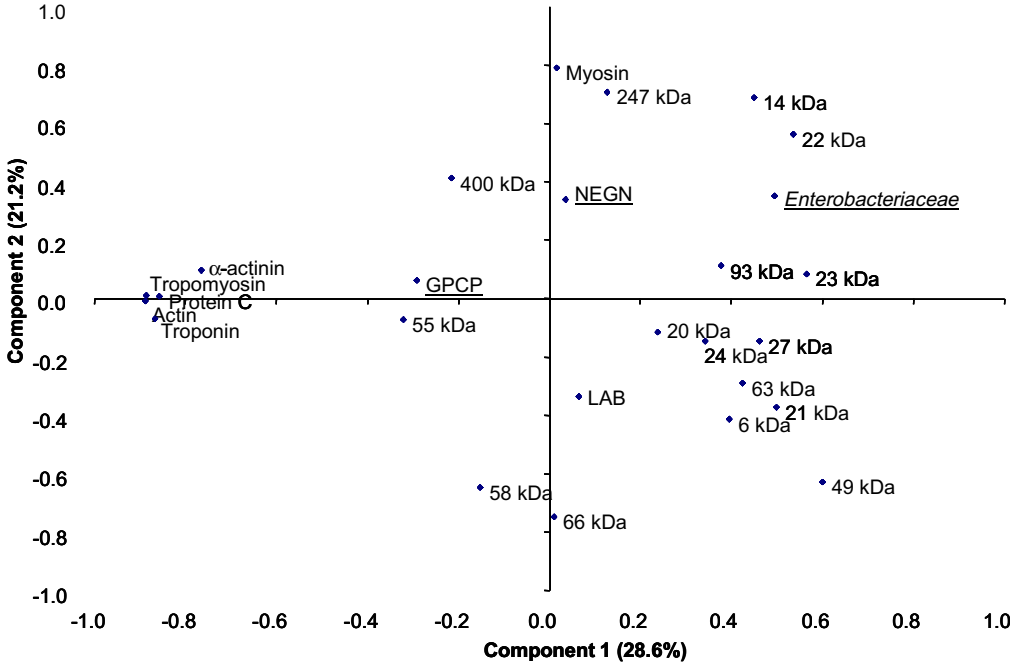

Fig. 1. Principal component analysis of the myofibrillar proteins and microbial counts: plane 1–2 of variable plot.

activity level between spoiled hams with 12 and 24 months of ripening. Indeed, peptides ranging from 50 to 100 kDa have been reported to result from myosin hydrolysis by endogenous enzymes in raw meat and cured meat products such as Iberian dry-cured ham (Benito et al., 2003; Córdoba et al., 1994a; Luccia et al., 2005).

In relation to the low ionic strength soluble fraction, the bands of 20, 31, 34, 42, 57, 72, and 93 kDa may be, respectively, myokinase, triosephosphate isomerase, phosphoglycerate mutase, phosphoglucomutase, pyruvate kinase, aldolase, and phosphorylase B according to their molecular weight. Also, the 36 kDa band could be made up of several sarcoplasmic proteins such as enolase, glyceraldehyde phosphate dehydrogenase, and creatine kinase (McCormick, Reeck, & Kropt, 1988). Moreover, the rest of the bands found in this low ionic strength soluble fraction are probably related to hydrolysis of this or the myofibrillar protein fraction. The results of the PCA of the low ionic strength soluble proteins and microbial counts are displayed in Fig. 2. These first two axes accounted, respectively for 45.2% and 15.3% of the total variance of the data. The most proteolytic microorganisms in this fraction were found among the Gram-negative microbial groups. The *Enterobacteriaceae* counts were associated with a lower intensity of the several bands defined by the first axis. Some of these may include sarcoplasmic proteins such as the 42, 36, 34, and 20 kDa bands. Also, NEGN counts showed a significant negative correlation with the 236, 85, 70, and 25 kDa peptides and a positive correlation with low molecular weight peptides such as 31 and 6 kDa, probably derived from the hydrolysis of the afore mentioned peptides. The proteolytic activity of NEGN was previously observed by Castillo et al. (1998) who mentioned in particular the production by *Aeromonas hydrophila* strains of proteases associated with pathogenic potential. The role of the GPCP in the degradation of the low ionic strength soluble proteins was quite similar to that of the NEGN (adjacent positions on the PCA), while the growth of LAB did not result in a greater hydrolysis of any particular protein or peptide. These findings are in agreement with reports in the literature of the scarce proteolytic activity of LAB on meat substrates (Leroy, Verluyten, & De Vuyst, 2006).

3.5. Relationship between the microbial population and free amino acids in the spoiled hams

The mean values of free amino acids in the spoiled hams ranged from 126 to 3222 mg/100 g dry matter (Table 4). For each free amino acid, the two spoiled batches showed similar percentages. These were different from those found in unspoiled hams for several amino acids (Martín et al., 2004). In particular, the basic amino acid arginine (Arg) was present at percentages higher than 20% in the spoiled hams studied while it is present at around 5% of total free amino acids in unspoiled Iberian dry-cured ham (Córdoba et al., 1994b; Martín et al., 2004). An inadequate proportion of such free amino acids not only can contribute to the generation of off-flavours, but also promote the generation of amines and other volatile compounds at undesirable concentrations. The amounts of the basic amino acids Arg and histidine (His) were increased by high counts of GPCP in spoiled hams studied (Table 4). This could explain, at least partially, the positive correlation between the high counts of this microbial group and the highest pH values in the spoiled hams. The amount of glutamine

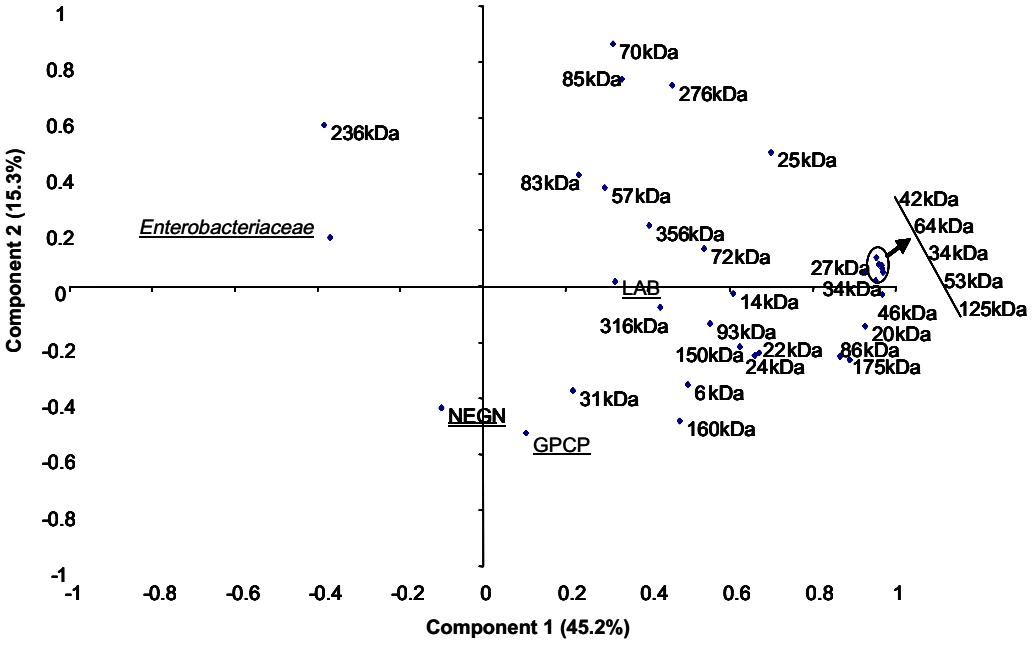

Fig. 2. Principal component analysis of the sarcoplasmic proteins and microbial counts: plane 1–2 of variable plot.

Table 4
Amino acid content of spoiled Iberian dry-cured hams and correlation with microbial counts

Amino acid	Spoiled hams				Correlations with microbial counts[b]			
	12 months		24 months					
	mg 100 g^{-1} (dry extract)	%	mg 100 g^{-1} (dry extract)	%	NEGN	GPCP	Ent.	LAB
Asp	298; 92[a]	2.53	126; 35	0.95	0.120	−0.007	0.203	−0.028
Glu	994; 143	8.42	1123; 158	8.48	−0.222	0.132	0.082	−0.052
Ser-Asn	329; 48	2.79	298; 45	2.25	0.082	−0.042	−0.153	−0.174
Gly	423; 56	3.59	518; 39	3.92	−0.281	0.313	−0.276	−0.045
Gln	301; 49	2.55	192; 118	1.45	−0.145	−0.079	0.331*	−0.097
His	487; 44	4.12	584; 45	4.41	−0.209	0.430*	0.212	−0.222
Thr	463; 33	3.92	504; 57	3.81	−0.501**	0.163	−0.071	−0.167
Ala	661;194	5.60	458; 178	3.46	−0.424*	0.321*	−0.012	−0.003
Pro	1013; 123	8.58	996; 90	7.53	−0.235	0.245	0.138	0.055
Arg	2775; 798	23.50	3222; 738	24.35	−0.234	0.510**	0.191	−0.179
Tyr	377; 36	3.19	317; 43	2.40	0.265	−0.014	0.117	−0.136
Val	624; 88	5.28	802; 82	6.06	−0.285	0.370*	0.037	−0.115
Met	232; 39	1.97	318; 30	2.40	−0.354*	0.281	0.108	−0.088
Ile	495; 72	4.19	645; 57	4.87	−0.340*	0.303	−0.148	−0.025
Leu	730; 98	6.18	997; 98	7.53	−0.338*	0.279	−0.067	−0.076
Phe	436; 54	3.69	547; 50	4.14	−0.278	0.234	−0.090	−0.075
Trp	326; 85	2.76	415; 75	3.13	−0.233	0.405*	0.097	−0.019
Lys	844; 97	7.14	1169; 121	8.83	−0.514**	0.044	0.106	0.061
Total	11808	100	13231	100				

[a] Mean and typical error of the free aminoacids values in the both batches of spoiled Iberian dry-cured hams.
[b] Ent: *Enterobacteriaceae*; NEGN: Non-enteric Gram-negative bacteria; GPCP:Gram-positive catalase-positive cocci. Regression coefficients of main effect.
* $P < 0.05$.
** $P < 0.001$.

(Gln), also seemed to increase with high counts of *Enterobacteriaceae*. The role of highly proteolytic microorganisms in the increase of several free amino acids such as Arg, Lysine (Lys), His, and Gln in dry-cured ham has been described elsewhere (Martín et al., 2002; Martín et al., 2004). On the other hand, the amounts of several amino acids were significantly negatively correlated with the higher NEGN counts in the spoiled hams, whereas the LAB counts had no relevant correlation with the concentration of any particular free amino acid in the spoiled

hams studied, confirming that LAB growth did not result in greater proteolytic activity.

3.6. Sensory analysis

With respect to the sensory analysis, the results of a principal component analysis performed on the sensory descriptor "spoilage level" and microbial counts obtained for each spoiled ham are displayed in Fig. 3. One observes that the samples formed three groups in relation with the first component, whereas the second component was related to the *Enterobacteriaceae* counts. The first group corresponded to strongly spoiled samples which mostly had counts greater than 2 log cfu g^{-1} of NEGN and GPCP. Several studies have found that the interaction between Gram-negative and Gram-positive bacteria can be important in food spoilage (Gram et al., 2002). For instance, inoculation with Gram-negative bacteria such as *Aeromonas* spp. did not cause spoilage of cold-smoked salmon, whereas the co-inoculation of these microorganisms together with Gram-positive bacteria such as *Brochothrix thermosphacta* and *Carnobacterium piscicola* produced spoilage off-odours (Joffraud et al., 2001). The second group, in a central position, corresponded to samples with a medium level of spoilage. Most of the samples with high counts of Gram-negative bacteria (NEGN or NEGN + *Enterobacteriaceae*) were included in this group. Finally, the third group of samples consisted of lightly spoiled hams. These mostly had counts greater than 2 log cfu g^{-1} of LAB, although they also included samples with high counts of NEGN and GPCP.

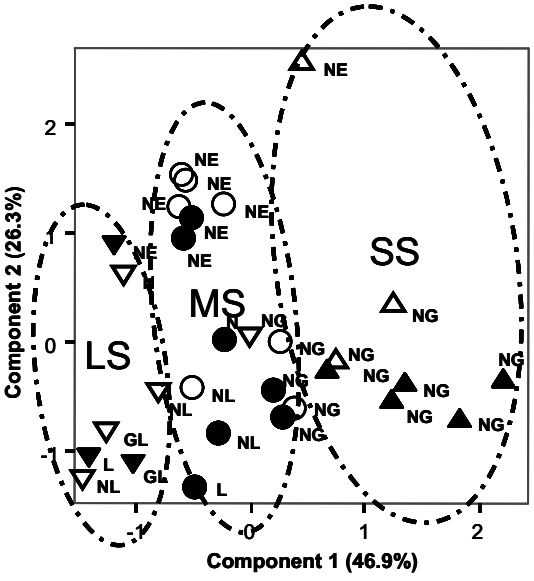

Fig. 3. Classification of ham samples depending on their spoilage level according to plane 1–2 of a factorial correspondence analysis. LS: Lightly spoiled samples ((▽) and (▼) symbols), MS: moderately spoiled samples ((○) and (●) symbols), SS: strongly spoiled samples ((△) and (▲) symbols). Samples of hams with 12 (open symbols) and 24 (filled symbols) months of ripening. Predominant microbial groups in the samples: L: LAB; N: NEGN; NL: NEGN+LAB; GL: GPCP + LAB; NE: NEGN + *Enterobacteriaceae*; NG: NEGN + GPCP.

4. Conclusion

There is evidence that deep spoilage of Iberian hams results from abnormal growth of the Gram negative bacteria NEGN and the highly proteolytic *Enterobacteriaceae*. Thus, appropriate programs to prevent contamination and growth of these microbial groups should be established in the meat industry to control the incidence of deep spoilage in the elaboration of dry-cured ham.

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