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Characterization and control of microbial black spot spoilage in dry-cured Iberian ham

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ABSTRACT

The presence of black spots on dry-cured Iberian ham surface is an alteration related to microbial population. Although it provokes important economic losses for the meat industry, the microorganisms responsible for this spoilage still remain unclear. The aim of this study was to identify the microorganisms involved in dry-cured Iberian ham black spot spoilage and to study the conditions affecting their growth. Several microbial strains were isolated from Iberian hams spoiled with black spots at the beginning of post-salting stage. However just one strain produced black coloration in both culture media and fat from Iberian ham. This strain was tentatively identified as *Pseudomonas fluorescens* by using the API 20 NE system and phylogenetic analyses based on the 16S rRNA and *car*A genes. It was able to grow and produce blackening at 5, 25, and 30 °C and with salt concentrations of 2 and 5% at 25 °C and with 2% NaCl at 5 °C in culture media. When it was inoculated in sterile pork fat tissues, grow and blackening were only detected in samples without added salt at water activity of 0.94 and 0.97. In order to control the incidence of the alteration temperature throughout salting and post-salting stages should be under 5 °C until salt reaches at least a concentration of 2% or water activity is reduced on the surface of dry-cured Iberian ham below 0.92.

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1. Introduction

Dry-cured Iberian ham is a traditional meat product obtained by a process which involves several months (18–24) of ripening. At the beginning of the process, the growth of undesirable microorganisms has to be prevented by adding salt and chilling until the stabilisation is reached by the gradual decrease of water activity throughout the ripening process. Thus at the end of the maturation period, hams usually present pH values ranging from 5.6 to 6.5 and water activity values down to 0.80 or even lower (Rodríguez et al., 1994). Although these ecological conditions do not favour microbial growth, several defects caused by spoilage microorganisms may happen throughout their long ripening period. One of these alterations only reported in Serrano dry-cured ham is the presence of black spots (Garriga, Ehrmann, Arnau, Hugas, & Vogel, 1998) which was first described by Hugas and Arnau (1987) on the surface of raw cured meat products. Black spots are localized very superficially on the hams mainly in the fat tissues and when the spot is removed by trimming, the contiguous muscular and connective tissues are unspoiled. Besides, the browned area is not characterized by an anomalous odour or texture. However, the presence of black spots on the surface of dry-cured Iberian ham is an important factor for consumer acceptance because this kind of meat products is mostly commercialized as whole pieces. As a result, black spot spoilage provokes important economic losses for dry-cured Iberian ham manufacturing industries.

Although some microorganisms, such as the Gram-negative bacterium *Carnimonas nigrificans*, have been related to black spot spoilage in some types of cured meat products (Garriga et al., 1998), the microorganisms responsible for this one in dry-cured Iberian ham still remain unclear. Despite the fact that water activity values on the ham surface decrease throughout the ripening process below the growth limit of most spoilage microbial population, the appearance of the first black spots has been observed at the beginning of the post-salting stage. It would be possibly due to the still high water activity values at this time that allow the growth of potential spoilage microorganisms. For that reason, to reduce the risk associated with these microorganisms in dry-cured Iberian ham, the environmental conditions should be carefully controlled

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during its maturation process. Firstly, it should be necessary to know what conditions throughout the dry-cured Iberian ham ripening may be used to avoid the growth of microorganisms responsible for black spots. These conditions should be based on temperature and salt concentration because they are the main preservative factors used during the maturation of dry-cured Iberian ham.

This study was primarily aimed to identify the microorganisms responsible for black spots on dry-cured Iberian ham by morphological, biochemical, and molecular methods. An additional objective was to study the environmental conditions affecting the growth of the microorganisms involved in this type of dry-cured ham spoilage.

2. Materials and methods

2.1. Sampling and microbiological analyses

Samples were taken from 5 dry-cured Iberian hams showing black spots at the beginning of post-salting stage. In order to perform microbial counts and isolates 10 g of the black spots were taken aseptically and homogenized in a Stomacher lab-blender with 90 mL of sterile peptone water (0.1% w/v) at room temperature. Appropriate serial decimal dilutions were made with the same diluent and 0.1 mL was spread onto the surface of Plate Count Agar (PCA) and Malt Extract Agar (MEA) (2% malt extract, 2% glucose, 0.1% peptone, 2% agar). Incubation was performed in the assayed media at two different temperatures (25 °C and 30 °C) for 48 h. Enumeration of total microorganisms in both media was then carried out. From the plates, representative colonies were selected according to morphological features (shape, colour, and size), including the strain able to produce blackening in the former culture media, purified by streaking, and stored frozen at −80 °C in Malt Extract Broth (MEB) (2% malt extract, 2% glucose, 0.1% peptone) plus glycerol (20% v/v). The selected isolates were routinely cultured on the same medium on which they had been isolated for further assays.

2.2. Morphological and biochemical identification

Each isolate was initially examined for cellular morphology under a microscope at 100× oil immersion objective and bacteria were tested by Gram stain. Subsequently microbial isolates were tentatively identified by using different API kits (bioMérieux, Marcy l'Etoile, France) in accordance with the specific manufacturer's instructions for each type of microorganism. Non-enteric Gram-negative bacteria were characterized by API 20 NE, Gram-positive cocci by API Staph, and yeasts by API 20C AUX. Examination of the strips was conducted after 24, 48, and 72 h. Interpretation of the results was carried out using the corresponding API identification table. The tests were repeated two times to determine the validity of the profiles generated.

For a preliminary identification of strain able to produce blackening in the culture media, catalase and oxidase activities were also performed using a hydrogen peroxide solution and *N*,*N*,*N*′,*N*′-Tetramethyl-p-phenylenediamine dihydrochloride (Sigma–Aldrich Co., St Louis, Missouri, USA), respectively. Oxidative and fermentative acid production (Hugh & Leifson, 1953) was also carried out for this strain.

2.3. Molecular identification

Only strain able to produce blackening in the culture media named S12N was further characterized by sequence analysis of the 16S rRNA and carbamoyl phosphate synthase small subunit (*car*A) genes.

2.3.1. DNA extraction

Strain S12N was grown in Brain Heart Infusion (BHI) broth for 24 h at 25 °C. One mL of culture was then used for the DNA extraction according to the method of Lawson, Gharbia, Shah, and Clark (1989), which includes the use of phenol/chloroform. Purified DNA was dissolved in 50 μL of sterile deionized water and stored at −20 °C until used for PCR reactions.

2.3.2. Sequencing of 16S rRNA and carA genes

The 16S rRNA gene of the bacterial strain S12N was amplified by PCR using two pairs of universal primers, A_{for} (5′ GGAGAGTTAGATCTTGGCTCAG 3′; nucleotides 6 to 27 of the *Escherichia coli* 16S rRNA gene) and C_{rev} (5′ AGAAAGGAGGTGATCCAGCC 3′; nucleotides 1542 to 1525 of the *E. coli* 16S rRNA gene) (Ntougias, Zervakis, & Fasseas, 2007), and PA (5′ AGAGTTTGATCCTGGCTCAG 3′; nucleotides 8 to 28 of the *E. coli* 16S rRNA gene) and PH (5′ AAGGAGGTGATCCAGCCGCA 3′; nucleotides 1542 to 1522 of the *E. coli* 16S rRNA gene) (Edwards, Rogall, Blöcker, Emde, & Böttger, 1989).

PCR amplification was performed with 50 μL reaction mixture, including 5 μL of 10× reaction buffer (10 mM Tris–HCl, pH 8.8, 50 mM KCl, and 0.1% Triton® X-100), 0.4 μM of each primer (Sigma–Aldrich Co.), 0.2 mM of each deoxynucleoside triphosphate (Roche Diagnostics, Indianapolis, Indiana, USA), 3 mM $MgCl_2$, 1 U of Taq polymerase (Finnzymes, Espoo, Finland), and 2 μL of DNA template. Negative controls containing sterile deionized water were included in place of template in each assay. Reactions were incubated in a Thermal Cycler of Bio Rad (mod. iCycler 170-8731). The PCR conditions for C_{rev} and A_{for} primers were 3 min of initial denaturation at 94 °C followed by 30 cycles of denaturation (1 min at 92 °C), annealing (1 min at 55 °C), and elongation (1.5 min at 72 °C), and a final extension of 5 min at 72 °C. The PCR conditions for PA and PH primers were as follows: initial denaturation of 3 min at 93 °C; 30 cycles consisting of 1 min at 92 °C, 1 min at 50 °C, and 2 min at 72 °C; final extension of 3 min at 72 °C.

The DNA extracted from strain S12N was also subjected to the amplification of the *car*A gene by using the primers and conditions reported by Hilario, Buckley, and Young (2004). A PCR product of about 700 bp was expected. The reaction mixture was performed as previously described for the 16S rRNA gene amplification.

After amplification, PCR products were visualized on 1% w/v agarose gels stained with ethidium bromide (0.5 μg/mL). These products were then purified using the MinElute® PCR Purification Kit according to the manufacturer's recommendations (QIAGEN, Hilden, Germany) and submitted for sequencing at Instituto de Biomedicina (CSIC, Valencia, Spain), with the same primers used in the amplification steps. Sequencing was performed from both the 5′ and the 3′ ends of each PCR product. The sequences were edited and assembled into a consensus sequence of the corresponding amplicon.

2.3.3. Sequence analysis

To determine the closest known relatives of the obtained 16S rRNA and *car*A gene sequences, searches were performed against the NCBI nucleotide nr and protein nr databases, respectively, with the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Owing to the fact that strain S12N was affiliated with the *Pseudomonas* genus and in order to analyse its phylogenetic position, both 16S rRNA and *car*A sequences were compared with those included in a previous work on the phylogenetic relationships among *Pseudomonas* (Hilario et al., 2004) (Table 1). Besides, additional *car*A gene sequences of other species and strains of

Table 1
GenBank database accession numbers for the *car*A and 16S rRNA genes of the *Pseudomonas* species used in the phylogenetic assays performed in this study.

Microorganism name	Strain number	GenBank accession number	
		*car*A	16S rRNA
Enterobacter sp.	638	CP000653.1	CP000653.1
P. aeruginosa	ICMP 8647^T	AJ414208.1	AJ308297.1
P. aeruginosa	PA7	CP000744.1	CP000744.1
P. aeruginosa	PAO1	AE004091.2	AE004091.2
P. agarici	ICMP 2656^T	AJ414209.1	AJ308298.1
P. aurantiaca	ICMP 6003^T	AJ414210.1	AJ308299.1
P. chlororaphis	ICMP 13613^T	AJ414212.1	AJ308301.1
P. chlororaphis subsp. *aureofaciens*	ICMP 13610^T	AJ414211.1	AJ308300.1
P. cichorii	ICMP 5707^T	AJ414213.1	AJ308302.1
P. entomophila	L48	CT573326.1	CT573326.1
P. flavescens	ICMP13539^T	AJ414214.1	AJ308320.1
P. fluorescens	ICMP 3512^T	AJ414220.1	AJ308308.1
P. fluorescens	LMG 14571		GU198119.1
P. fluorescens biotype A	ICMP 13622	AJ414215.1	AJ308303.1
P. fluorescens biotype B	ICMP 13619	AJ414216.1	AJ308304.1
P. fluorescens biotype C	ICMP 13624	AJ414217.1	AJ308305.1
P. fluorescens biotype F	ICMP 13616	AJ414218.1	AJ308306.1
P. fluorescens biotype G	ICMP 13621	AJ414219.1	AJ308307.1
P. fragi	DSM 3456	DQ647053.1	
P. lundensis	DSM 6252	DQ647052.1	
P. marginalis	ICMP 3553^T	AJ414221.1	AJ308309.1
P. mendocina	ICMP 13540^T	AJ414222.1	AJ308310.1
P. mendocina	ymp	CP000680.1	CP000680.1
P. poae	LB-Z1		AB495132.1
P. putida	DSM 50222	EF363547.1	
P. putida	W619	CP000949.1	CP000949.1
P. putida	ICMP 2758	AJ414225.1	AJ308313.1
P. putida biotype A	ICMP 13629	AJ414223.1	AJ308311.1
P. putida biotype B	ICMP 13630	AJ414224.1	AJ308312.1
P. reactans	PSR2		GQ354529.1
P. reactans	CAI-4		DQ257418.1
P. resinovorans	ICMP 13541^T	AJ414226.1	AJ308314.1
P. sp. *phi9*	phi9		GU233953.1
Pseudomonas sp. *G*	G	EU162747.1	
P. sp.	W15Feb33		EU681016.1
P. stutzeri	A1501	CP000304.1	CP000304.1
P. stutzeri	ICMP 12561^T	AJ414227.1	AJ308315.1
P. synxantha	IHB B 1322		GU186110.1
P. synxantha	DSM 13080		AF267911.1
P. syringae	ICMP 3023^T	AJ414228.1	AJ308316.1
P. syringae pv. phaseolicola	1448A	CP000058.1	CP000058.1
P. syringae pv. tabaci	ATCC 11528	ACHU02000114.1	
P. tolaasii	ICMP 12833^T	AJ414229.1	AJ308317.1

Pseudomonas were included, as well as some of the best hits of the 16S rRNA gene in the blastn search (Table 1).

The *car*A amino acid sequences were aligned using ClustalW version 2.0.9 (Thompson, Higgins, & Gibson, 1994) under the default parameters. Amino acid alignments were transcribed to the corresponding nucleotide ones with RevTrans v1.4 (Wernersson & Pedersen, 2003).

The 16S rRNA nucleotide sequences were aligned using mothur (Schloss et al., 2009) and the aligned sequences of the Greengenes 'Core Set' as template alignment (DeSantis et al., 2006). The closest template for each sequence was found using 9-mer searching and the pairwise alignment between the sequences and the templates was made using the Gotoh algorithm.

Both ends of each multiple alignment were trimmed to the following final sizes: *car*A 624 positions, 16S rRNA gene 1371 positions.

Phylogenetic trees were obtained by maximum likelihood with the program PhyML v3.0 (Guindon & Gascuel, 2003) using the general time reversible (GTR) model of nucleotide substitution and 1000 bootstrap replicates.

2.4. Growth at different temperatures and NaCl concentrations in culture media

In order to evaluate the optimal temperature of strain S12N to grow and produce browning, it was inoculated on MEA at 5, 25, 30, and 37 °C. Growth was evaluated by observing a full biomass development along the streaking point. Browning was scored as positive if black colonies were observed. The microorganism was also inoculated in other two culture media (PCA and BHI broth) at the temperatures described above. Growth and browning production were considered positive in liquid media when turbidity and black colour appeared, respectively.

Furthermore the effect brought by different salt concentrations (0, 2, 5, 10, and 15% w/v NaCl), which can be encountered on the dry-cured Iberian ham surface during its maturation process, was also tested in MEB at 5 and 25 °C. For this, commercially available pre-sterilized flat-bottomed 96-well microtiter plates (Bioster, Bastia di Rovolon, Italy) were used. At first, 140 μL of sterile MEB containing the tested amounts of salt were transferred into selected wells of a microtiter plate under aseptic conditions. Each well was then inoculated with 10 μL of an overnight culture of strain S12N in a concentration of about 10^6 cfu/mL. Wells filled only with MEB containing each of the assayed NaCl concentrations were used as controls. The plates were placed in an incubator at 5 and 25 °C for 6 days. Absorbance was measured on a microtiter plate reader (Merck KGaA, Darmstadt, Germany) every 24 h at a wavelength of 595 nm. All the assays were performed in triplicate. The results were expressed as the average absorbance for each inoculated MEB supplemented with the tested amounts of salt regarding the means of absorbance data obtained to each corresponding non inoculated one. Furthermore, growth and blackening generation were visually monitored and they were considered positive as described above for liquid culture media.

2.5. Growth in fat of Iberian ham

In order to reproduce the black spot spoilage, fat tissues of non salted Iberian hams were inoculated with strain S12N and incubated at different temperature and water activity conditions, and NaCl concentrations. For this, sterile 20 g samples were obtained in a laminar flow cabinet Bio Flow II (Telstar, Tarrasa, Spain) from fat tissues removed from Iberian ham immediately after slaughter. To simulate the conditions of hams during the post-salting stage, saturated solutions of potassium sulphate and potassium nitrate were placed in sterile receptacles in order to reach water activity values of 0.97 and 0.94, respectively. Three samples of fat tissue were then placed in each one. Sterile NaCl was also added in order to reach 2 and 5% w/w NaCl in the samples. A batch with no added NaCl was also incubated for each water activity value. Thus, a total of 6 batches were obtained and inoculated with 1 mL containing approximately 10^6 cfu/mL of the strain S12N. Other 6 batches were not inoculated and maintained as non inoculated controls.

During the first 15 days, all the above batches were incubated at 10 °C and then were taken at 15 °C for other 15 days. Finally they were kept at 20 °C during the last 15 days of incubation. Blackening appearance was visually monitored every 24 h. Besides, triplicates samples of 10 g each from every batch were taken and the viable number of S12N determined at the end of the incubation period (45 days) on MEA plates.

3. Results

3.1. Microbial counts and identification using phenotypic methods

In samples from black spots spoiling dry-cured Iberian hams, the microbial counts in MEA were higher than those obtained in

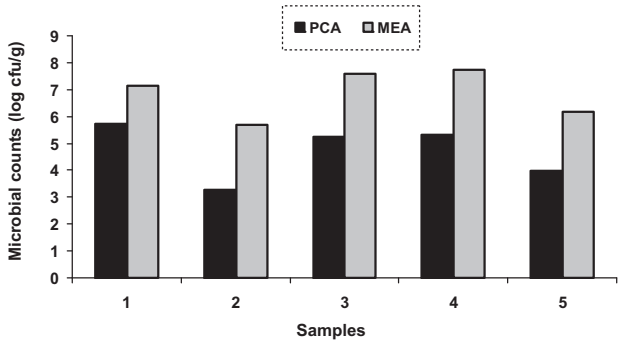

Fig. 1. Microbial population enumerated on Plate Count Agar (PCA) and Malt Extract Agar (MEA) of 5 dry-cured Iberian ham samples with black spot spoilage.

PCA, being at levels of about 10^7 cfu/g and 10^5 cfu/g respectively (Fig. 1).

Characterization analyses by morphological and biochemical tests of the microbial strains obtained in the former culture media showed *Staphylococcus xylosus* as the most frequently isolated species in black spot samples (Table 2). Other strains were found at lower proportion such as *Moraxella* spp., *Rhodotorula* spp., and a yeast strain characterized as it by morphological examination and misidentified by API 20C AUX (Table 2). In addition, a low percentage (6.25%) of the isolates showed blackening in MEA at 25 °C (Table 2). These isolates, named S12N, were found to be Gram-negative, catalase and oxidase positive rod, and showed oxidative metabolism on Hugh Leifson medium.

From the results of the biochemical characterization determined by means of API 20 NE system, strain S12N was tentatively characterized as *Pseudomonas fluorescens* (Table 2). Further characterization of this strain was achieved by 16S rRNA and *car*A gene sequencing.

3.2. Identification by 16S rRNA and carA gene sequencing

Almost the complete sequence of the 16S rRNA (1444 bp) of the strain S12N was used to its taxonomic affiliation. According to a blast search, the strain S12N exhibited the same sequence identity (99%) with the 16S rRNA gene sequences of several strains of *Pseudomonas,* including strains within *P. fluorescens*, *Pseudomonas synxantha*, and *P. reactans*, among others.

Since the result obtained by means of 16S rRNA sequence was not sufficient to identify the strain S12N at species level, its *car*A gene sequence was obtained (621 bp) and analysed with a blast search which displayed a sequence identity of 100% with the *P. fluorescens* ICMP 13622 *car*A gene.

The phylogenetic position of the strain S12N was then analysed with the 16S rRNA and *car*A genes (Fig. 2). Some discrepancies between the two phylogenies were found in the relationships among the strains included in the analysis and several *Pseudomonas* species were seen as polyphyletic. Also, more nodes in the 16S rRNA phylogeny got low bootstrap support and were considered as unresolved, as expected given the higher level of sequence conservation of this marker.

The strain S12N was placed in an unresolved group with other *Pseudomonas* strains in the two phylogenies (Fig. 2). For the *car*A gene, its closest relatives were two strains of *P. fluorescens* (Fig. 2a). However, all the strains of *P. fluorescens* did not form a monophyletic group either in this phylogeny or the one for the 16S rRNA. For the 16S rRNA gene, the strain S12N was closely related to several *Pseudomonas* species like *P. fluorescens*, *P. synxantha*, and *Pseudomonas reactans* (Fig. 2b). From these results and taking into account the previous biochemical identification, it would be reasonable to characterize as *P. fluorescens* the Gram-negative bacterium responsible for black spots in the dry-cured Iberian hams.

Table 2
Morphological and biochemical identification of microbial strains isolated from black spots spoiling dry-cured Iberian hams by API kits. The occurrence of identified strains is shown.

Morphological characteristics	API Identification[a]	Percentage of isolation (%)
Gram-positive cocci	*Staphylococcus xylosus*	68.75
Gram-negative rods	*Pseudomonas fluorescens*	6.25
Gram-negative rods	*Moraxella* spp.	6.25
Yeasts	*Rhodotorula* spp.	6.25
Yeasts	—[b]	12.50

[a] Gram-positive bacteria were tentatively identified by API Staph, Gram-negative bacteria by API 20 NE, and yeasts by API 20C AUX.
[b] Misidentified strains.

3.3. Growth of strain S12N at different conditions of temperature and NaCl concentration in culture media

When strain S12N was inoculated at different temperatures, no growth was observed at 37 °C in any of the three assayed culture media (Table 3). On the contrary, it grew and produced blackening at 5, 25, and 30 °C in all of them, except at the last temperature in PCA since black colonies were not observed. The fastest growth and blackening production were detected in BHI broth and MEA at 25 °C, though the blackening appeared earlier in MEA than in the other media at the same temperature value (Table 3). At the rest of assayed temperatures the behaviour of the bacterium was similar but both growth and browning were reached later. Furthermore, they appeared earlier at 30 °C than at 5 °C (Table 3).

On the other hand, in all of the cases it was observed that the browning generation of strain *P. fluorescens* S12N began with brown colour which became black after a few hours.

Afterwards, effects of salt concentration on *P. fluorescens* S12N growth and the subsequent blackening production were analysed by means of microtiter plates and absorbance measurements during 6 days at 25 and 5 °C.

Considering 24 h of incubation at 25 °C, *P. fluorescens* S12N growth was detected in both MEB without added NaCl and with 2% NaCl because of an increase in the absorbance values (Fig. 3a) and the visually observed turbidity (Table 4). However, blackening production was only reached in absence of NaCl (Fig. 3a, Table 4) being detected as a higher absorbance value than in MEB with 2% NaCl (Fig. 3a) and a visualized black colour in the liquid medium (Table 4). After 48 h of incubation, blackening appeared in MEB with 2% NaCl (Table 4) and the tested strain was able to grow with a concentration of 5% NaCl showing a lower absorbance value than MEB without salt and with 2% NaCl at 24 h of incubation (Fig. 3a). No significant changes were detected in either microbial growth or blackening generation after 6 days of incubation (Fig. 3a). Therefore no growth and black colour formation were observed at the salt concentrations of 10% and 15%. Furthermore *P. fluorescens* S12N was very slow to produce black colour in MEB supplemented with 5% NaCl being almost imperceptible at the end of the incubation period (Fig. 3a, Table 4). The highest absorbance value and consequently the highest level of browning were reached in the MEB without added salt (Fig. 3a).

Regarding to 5 °C, none of the assayed amount of salt allowed *P. fluorescens* S12N growth at 24 h and 48 h of incubation (Table 4). However, it was detected at 3 days of incubation in MEB without added salt and with 2% NaCl because of an increase in the absorbance values (Fig. 3b) and a visualized turbidity in the liquid

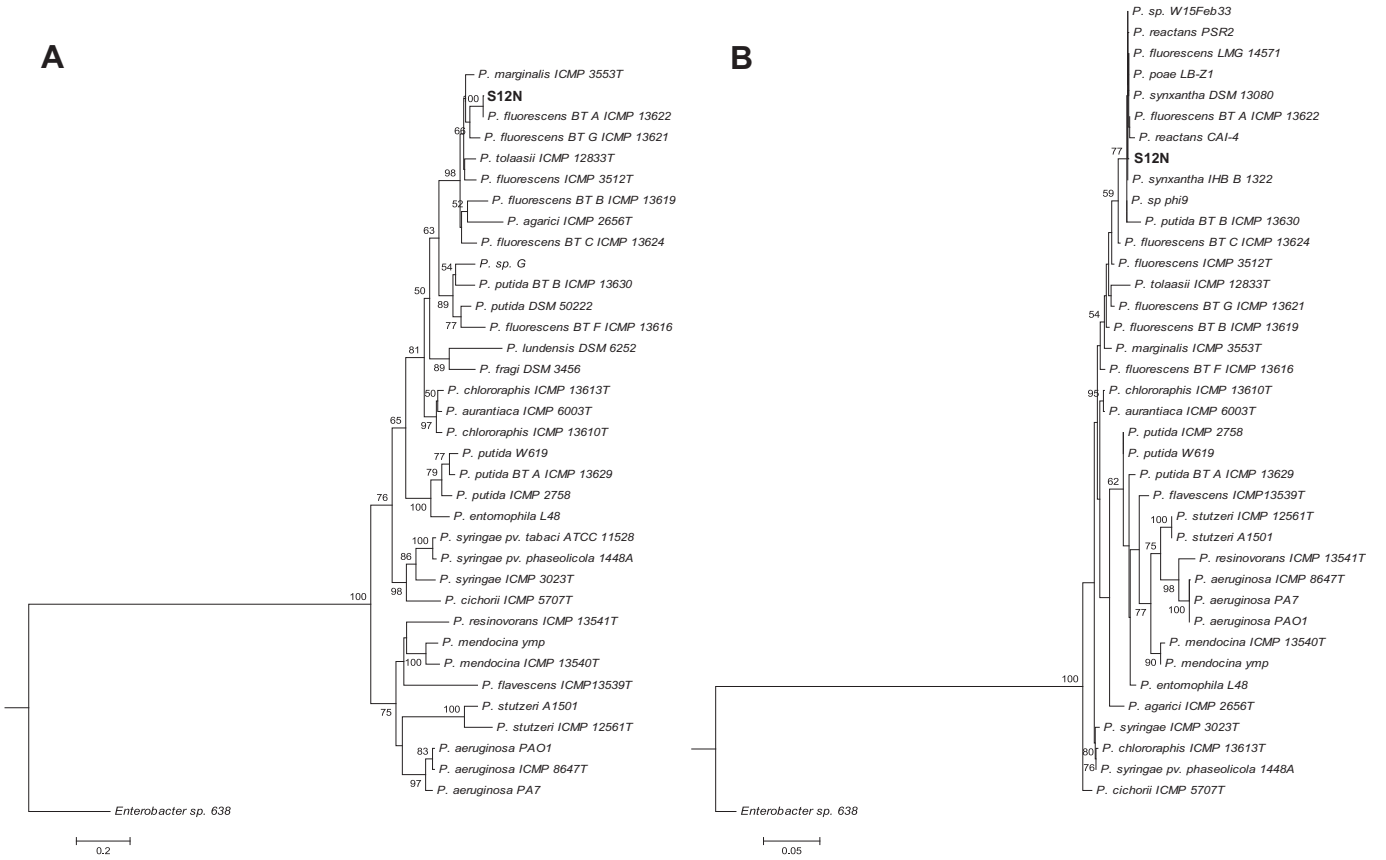

Fig. 2. Maximum likehood phylogenetic trees of *Pseudomonas fluorescens* S12N isolated from black spots in dry-cured Iberian ham and *Pseudomonas* strains using *car*A (A) and 16S rRNA (B) genes. Bootstrap percentages of 50% or more are indicated at the branch points. Enterobacter was included as an outgroup. Strain and accession numbers are shown in Table 1.

medium (Table 4). Blackening appeared at 3 and 6 days of incubation in the media abovementioned, respectively (Fig. 3b, Table 4). At the end of the incubation period, the unique detected change was the appearance of S12N growth in MEB with 5% NaCl even though it was not very intense (Fig. 3b, Table 4). As it was obtained at 25 °C, the greatest absorbance value and the resulting greatest level of browning at 5 °C were obtained in the medium without NaCl (Fig. 3b).

Table 3
Growth and browning behaviour of *Pseudomonas fluorescens* S12N using the indicated culture media and incubation temperatures. Both characteristics were visually monitored.

		Culture media[a]		
		Malt Extract Agar	Plate Count Agar	Brain Heart Infusion broth
Growth	5 °C	7[b]	7	6
	25 °C	1	2	1
	30 °C	5	5	3
	37 °C	n.d.[c]	n.d.	n.d.
Blackening	5 °C	11	12	7
	25 °C	2	3	3
	30 °C	7	n.d.	7
	37 °C	n.d.	n.d.	n.d.

[a] Growth and blackening production were considered positive in Malt Extract Agar and Plate Count Agar when a full biomass and black colonies were observed, respectively. In Brain Heart Infusion broth growth and blackening generation were considered positive if turbidity and black colour were developed, respectively.
[b] Time at which growth and browning production were reached are expressed in days.
[c] n.d.: not detected.

3.4. Growth of strain S12N in fat of Iberian ham

When strain S12N was inoculated in sterile fat of Iberian ham and incubated at different conditions of temperature, water activity, and NaCl levels, black spots were observed in the batches without added salt (Fig. 4a) at 2 days of incubation period when water activity was 0.97 and after 3 days of incubation at 0.94 of water activity. In both cases, blackening was detected at 10 °C and it was progressively developed in the fat tissues. Blackening was not detected in any of the salted samples after incubation at 10, 15, and 20 °C. Non inoculated controls remained unspoiled in salted and non salted fat samples (Fig. 4b).

When *P. fluorescens* S12N counts were performed on MEA after incubation period of 45 days, black colonies were obtained in fat tissues without added NaCl at both tested water activity values (Table 5). Furthermore, the obtained S12N levels were higher than those inoculated at the beginning of the incubation period. *P. fluorescens* S12N was no detected in any of the salted fat tissues at the end of incubation period (Table 5). Strain S12N was not detected in the control batches either (Table 5). This finding matched up with the abovementioned results on the black spot visualized in fat tissues.

4. Discussion

Microbial counts found in black spots of dry-cured ham at the beginning of processing (post-salting stage) at levels of 10^5-10^7 cfu/g in the two assayed culture media were in those levels reported in unspoiled dry-cured Iberian hams at the same ripening stage

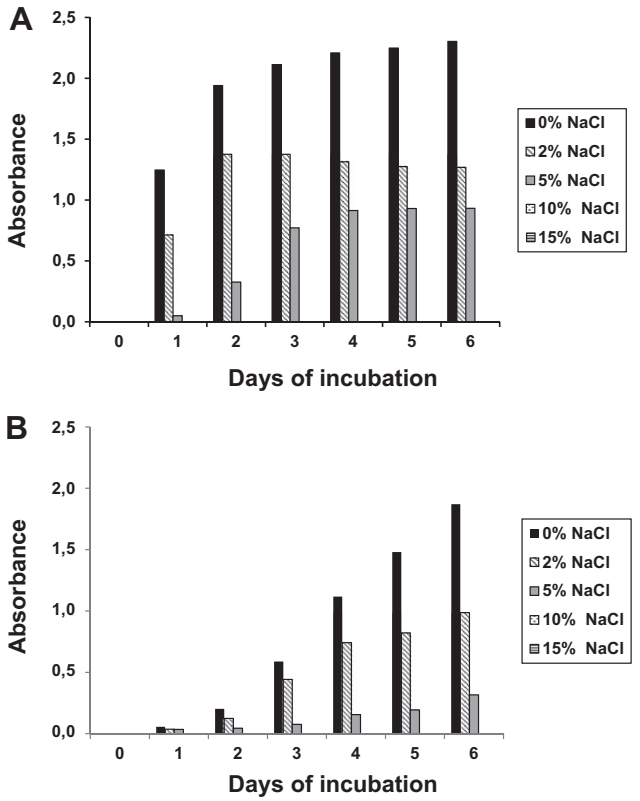

Fig. 3. Growth curves of *Pseudomonas fluorescens* S12N in the presence of several salt concentrations at 25 °C (A) and 5 °C (B). Absorbance was measured at 595 nm every 24 h.

Table 4
Effect of temperature and NaCl concentration on *Pseudomonas fluorescens* S12N growth and browning production. Both characteristics were monitored by visual observation and absorbance measure at 595 nm.

		Days of incubation						
		0	1	2	3	4	5	6
		25 °C						
Growth	0% NaCl	−[a]	+	+	+	+	+	+
	2% NaCl	−	+	+	+	+	+	+
	5% NaCl	−	−	+	+	+	+	+
	10% NaCl	−	−	−	−	−	−	−
	15% NaCl	−	−	−	−	−	−	−
Blackening	0% NaCl	−	+	+	+	+	+	+
	2% NaCl	−	−	+	+	+	+	+
	5% NaCl	−	−	−	−	−	−	+
	10% NaCl	−	−	−	−	−	−	−
	15% NaCl	−	−	−	−	−	−	−
		5°C						
Growth	0% NaCl	−	−	−	+	+	+	+
	2% NaCl	−	−	−	+	+	+	+
	5% NaCl	−	−	−	−	−	−	+
	10% NaCl	−	−	−	−	−	−	−
	15% NaCl	−	−	−	−	−	−	−
Blackening	0% NaCl	−	−	−	−	+	+	+
	2% NaCl	−	−	−	−	−	−	+
	5% NaCl	−	−	−	−	−	−	−
	10% NaCl	−	−	−	−	−	−	−
	15% NaCl	−	−	−	−	−	−	−

[a] Growth and browning production are scored as +, positive; −, negative. Growth and blackening generation were considered positive in Malt Extract Broth by visual observation if turbidity and black colour were developed, respectively, and spectrophotometrically when absorbance values were above 0.3 and 1.0, respectively.

(García, Martín, Timón, & Córdoba, 2000; Núñez, Rodríguez, Córdoba, Bermúdez, & Asensio, 1996). In addition, most of the isolated strains were characterized as microorganisms usually found on the surface of unspoiled dry-cured hams such as *S. xylosus* or *Rhodotorula* spp. (Núñez et al., 1996; Rodríguez, Núñez, Córdoba, Bermúdez, & Asensio, 1996). *Moraxella* spp. has been previously described in meat stored under refrigeration (Li, Zhou, Xu, Li, & Zhu, 2006) but not in dry-cured ham. The misidentified yeast strain may belong to *Debaryomyces hansenii*, species usually found on the surface of dry-cured Iberian ham (Andrade, Rodríguez, Casado, Bermúdez, & Córdoba, 2009; Núñez et al., 1996) and not included in the API 20C AUX system. None of the aforementioned microbial species has been reported as causing microbial spoilage in dry-cured Iberian hams. *S. xylosus* and *D. hansenii* have been even reported as appropriate for being used as starter cultures (Andrade, Córdoba, Casado, Córdoba, & Rodríguez, 2010; Rodríguez, Núñez, Córdoba, Bermúdez, & Asensio, 1998). Regarding the Gram-negative bacteria identified as *Moraxella* spp., references about its presence or its contribution either positive or negative ripening process of dry-cured meat products are scarce.

The Gram-negative strain, which was identified as *P. fluorescens* S12N according to the API 20 NE was able to produce blacking in one of the assayed culture media and also in fat from Iberian hams where black spots showed the same aspect that those found in spoiled dry-cured Iberian ham.

This microbial species has not been reported in dry-cured ham, but it has been related to spoilage of fresh meat (Kröckel, 2009). Therefore, although this species represented only about 6% of the total microbial population found in black spot samples in the present work, it may be responsible for this alteration.

The identification result obtained using the conventional biochemical assay for strain S12N was compared to the results of identification based on molecular techniques. For this, sequence analysis of the 16S rRNA gene was chosen since it is widely employed for molecular identification of bacteria and used as a phylogenetic marker. By using this methodology, the strain S12N was identified as *Pseudomonas* spp. but the species could not be assigned because it showed identical sequence similarity with several *Pseudomonas* species. This is in agreement with other researchers who have found that the 16S rRNA gene does not always differentiate satisfactorily at species level within the *Pseudomonas* genus (Ercolini, Russo, Torrieri, Masi, & Villani, 2006; Yamamoto et al., 2000). As an example, great efforts have been devoted to characterizing *Pseudomonas* spp. associated with milk spoilage (Dogan & Boor, 2003; He, Dong, Lee, & Li, 2009; Wiedmann, Weilmeier, Dineen, Ralyea, & Boor, 2000). It seems that the 16S rRNA gene is limited in its use for the identification of *Pseudomonas* isolated from the meat ecosystem too.

Alternatively, several gene sequences have been explored as targets for PCR-based differentiation of common *Pseudomonas* spp. causing food spoilage (Ercolini et al., 2006; Scarpellini, Franzetti, & Galli, 2004). In the present study, *car*A gene was chosen because it has been described as supportive for *Pseudomonas* species differentiation (Hilario et al., 2004). The main problem of the use of *car*A gene is that fewer sequences are available in public databases. Using this gene, the closest relative of the strain S12N was identified as *P. fluorescens*.

On the other hand, the results derived from molecular techniques were consistent with those from the API 20 NE system, mainly when *car*A gene sequences and biochemical identification were related. Regarding the comparison with 16S rRNA, Bosshard et al. (2006) obtained similar results because the sequences of an unidentified isolate showed 100% identity to sequences of *P. fluorescens* and *Pseudomonas jessenii*, whereas API 20 NE identified it as *P. fluorescens*. This commercial API system could be

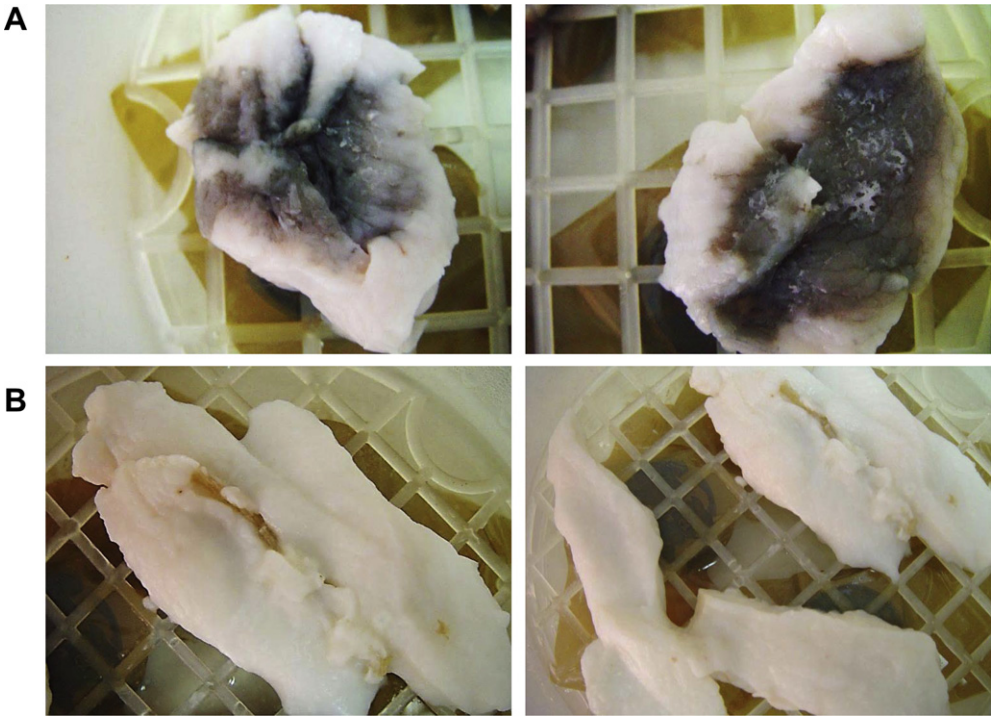

Fig. 4. Examples of black spot spoilage formation in fat tissues from non salted Iberian ham artificially contaminated with *Pseudomonas fluorescens* S12N after 15 days of incubation period at 10 °C (A). Alteration was not detected in either salted at 2% NaCl or non inoculated fat samples after 15 days of incubation at 10 °C (B).

considered appropriate for *Pseudomonas* strains since it has provided a good identification of dairy *Pseudomonas* isolates to the species level as well (Wiedmann et al., 2000). From the results of this study, both biochemical and molecular methods should be considered in the identification of microorganisms involved in black spot spoilage in dry-cured Iberian meat products. It is in accordance with Franzetti and Scarpellini (2007) who considered that a correct identification and characterization of some *Pseudomonas* species can only be achieved by combining cultural, biochemical, and molecular techniques.

P. fluorescens is a psychrotrophic bacterium which can be found in a variety of food related environments (Sillankorva, Neubauer, & Azeredo, 2008). It has been traditionally recognized as milk spoiler and it is one of the most representative *Pseudomonas* species involved in browning in vegetables spoilage (Franzetti & Scarpellini, 2007). Besides, it has been also involved in spoilage of meat even during storage at low temperatures (Ercolini et al., 2007) because of its psychrotrophic character. Recently, Kröckel (2009) has isolated *P. fluorescens* from refrigerated spoiled pork and beef meat with black spots. In addition, *P. libaniensis* has been reported as responsible for blue spots in raw hams (Cantoni, Stella, Comi, & Cocolin, 2001). However, *Pseudomonas* spp. or *P. fluorescens* have not been previously described as responsible for black spot formation in dry-cured meat products, since the microorganism involved in this alteration was identified as *Carnimonas nigrificans* (Garriga et al., 1998).

Table 5
S12N counts on Malt Extract Agar obtained for fat samples of Iberian ham after 45 days of incubation (15 days at 10 °C, following by 15 days at 15 °C, and an additional period of 15 days at 20 °C).

Water activity[a]	%NaCl	S12N counts (cfu/g)	
		Control samples[b]	Inoculated samples[c]
0.97	0	n.d.[d]	$(1.6 \pm 0.3) \times 10^8$
	2	n.d.	n.d.
	5	n.d.	n.d.
0.94	0	n.d.	$(7.6 \pm 6.7) \times 10^7$
	2	n.d.	n.d.
	5	n.d.	n.d.

[a] Saturated solutions of potassium sulphate and potassium nitrate were used to reach water activity values of 0.97 and 0.94, respectively.
[b] Non inoculated fat tissue samples.
[c] Fat samples inoculated with approximately 10^6 ufc/ml of the strain S12N at the beginning of this period.
[d] n.d.: black colonies were not detected.

On the other hand, since both growth and browning generation were observed faster in MEA, which is selective for yeasts and moulds, and BHI broth than in PCA, these culture media should be proposed as appropriate for isolating and doing routine cultures of *P. fluorescens* involved in black spot spoilage of dry-cured Iberian ham. In addition, the visualized behaviour of blackening generation in all the tested culture media was very similar to that observed in dry-cured Iberian ham as the colonies or the liquid medium were first brown and became black in a few hours.

In this study, an experimental design was employed to assess the effects of temperature and salt concentration on *P. fluorescens* S12N growth and browning generation. Many authors have studied the growth ability of *P. fluorescens* related to meat industry, but most works have been carried out in chilled raw meat or in media simulating raw meat (Lebert, Baucour, Lebert, & Daudin, 2005; Lebert, Begot, & Lebert, 1998). The temperatures and NaCl levels chosen in the present work were those usually found throughout the dry-cured Iberian ham ripening.

P. fluorescens S12N showed growth and browning production at 5, 25, and 30 °C. Besides, levels of salt higher than 5% NaCl inhibited both growth and browning appearance at 25 °C. Growth ability of *P. fluorescens* in 5% NaCl has been previously described by Narbad and Gasson (1998). These authors obtained similar results to those found in the present research observing that *P. fluorescens* did not grow at 37 °C and the growth was more efficient at 25 °C than at 30 °C.

When the strain S12N was inoculated in MEB no growth and blackening were observed at NaCl concentrations of 10% and 15% which could be considered as equivalent water activity values of about 0.92 and 0.88, respectively (Neidleman & Laskin, 1997).

Nevertheless when the microorganism was inoculated in pork fat tissues from Iberian hams, salt concentrations of 2% or higher, combined with water activity of 0.97 and 0.94, resulted in its reduction and consequently blackening did not appear. According with these findings, water activity below 0.92 seems to be appropriate to prevent black spots spoilage. Thus when the environmental conditions are adequately controlled throughout the maturation process of dry-cured Iberian hams, the water activity ranges usually from 0.89 to 0.91 in their surfaces at the post-salting stage (Rodríguez et al., 1994) and consequently it should be sufficient to avoid the growth of microorganism involved in black spot alteration. Furthermore it should be necessary to ensure the suitable water activity on the whole surface of dry-cured Iberian hams during the salting stage and at the beginning of post-salting since the presence of areas with high water activity may allow the growth of strain S12N and the subsequent spoilage. In this sense, the current tendency consisting of reducing salt on hams with healthy purposes may generate that not enough salt should be distributed for the surface of hams, which favours growth of this microorganism and consequently the formation of black spots. Thus, in order to control this spoilage it is essential to achieve a homogenous and sufficient distribution of NaCl during salting period that guarantees at least concentrations of 2% in the whole ham surface.

Due to the fact that at 5 °C in MEA with 5% NaCl a weak ability of S12N for growing was observed and blackening was not appeared, temperature should be an important factor to be carefully controlled during salting and post-salting stages as well. Thus temperature of 5 °C or bellow may be ensured during these periods of the dry-cured Iberian ham ripening.

In addition, significant contaminations by *Pseudomonas*, including *P. fluorescens*, can occur during the preparation of raw hams due to the common presence of this species in the slaughterhouse or inadequately sanitized surfaces of storage, transporting equipments, and cutting process of carcasses. Thus, an exhaustive hygiene program should be taken in order to avoid contamination with the microorganism involved in black spot alteration.

5. Conclusion

In the present work it was concluded that *P. fluorescens* was the microorganism responsible for black spot spoilage in dry-cured Iberian hams. To control the incidence of this alteration an adequate combination of temperature, salt, and water activity should be ensured. This should consist of a temperature below 5 °C until salt reaches at least a concentration of 2% or water activity is reduced on the surface of the hams below 0.92.

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