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Characterisation and detection of spoilage mould responsible for black spot in dry-cured fermented sausages

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ABSTRACT

Moulds responsible for black spot spoilage of dry-cured fermented sausages were characterised. For this purpose, samples were taken from those dry-cured fermented sausages which showed black spot alteration. Most of the mould strains were first tentatively identified as *Penicillium* spp. due to their morphological characteristics in different culture conditions, with one strain as *Cladosporium* sp. The *Cladosporium* strain was the only one which provoked blackening in culture media. This strain was further characterised by sequencing of ITS1-5.8S-ITS2 rRNA and *β-tubulin* genes. This mould strain was able to reproduce black spot formation in dry-cured fermented sausage 'salchichón' throughout the ripening process. In addition, a specific and sensitive real-time PCR method was also developed to detect *Cladosporium oxysporum* responsible for the black spot formation in sausages. This method could be of great interest for the meat industry to detect samples contaminated with this mould before spoilage of product avoiding economic losses for this sector.

1. Introduction

Dry-cured fermented meat sausages are produced through the world (Fonseca, Ouba, Franco, & Cárballo, 2013; Krkić et al., 2013; López-Díaz, Santos, García-López, & Otero, 2001; Tabanelli et al., 2012). The environmental conditions in the manufacturing rooms for dry-cured fermented sausages production favour microbial growth, especially fungi, on the surface of products (Comi, Orlic, Redzepovic, Urso, & Iacumin, 2004; Mizakovà, Pipovà, & Turek, 2002). Some mould strains may produce undesirable effects on the quality of these products such as off-flavours, colour of conidia and floccose mycelium on the casing (Ludemann, Greco, Rodríguez, Basílico, & Pardo, 2010), with one of these undesirable effects being the formation of black spots. Black spots as a result of microbial growth in different meat products such as dry-cured "Serrano" and Iberian dry-cured hams have been previously reported (Andrade, Rodas, Durbán, Moya, & Córdoba, 2012; Garriga, Ehrmann, Arnau, Hugas, & Vogel, 1998; Hugas & Arnau, 1987). This alteration has not hitherto been reported in dry-cured fermented sausages, although lately it is usually found in this kind of products in the meat industry. Black spots are localized very superficially on the casing of the sausage. The spoiled and browned area in the sausages is not characterised by an anomalous odour or texture. However, the presence of black spots on the surface of dry-cured fermented sausages could be an important factor for consumer acceptance when this kind of meat product is commercialized as whole pieces. As a result, black spot spoilage may provoke important economic losses for dry-cured sausages manufacturing industries.

To control black spots in dry-cured fermented sausages it is first necessary to characterise micro-organisms involved in this alteration. In "Serrano" dry-cured ham black spots were caused by growth of *Carnimonas nigrificans* (Garriga et al., 1998; Hugas & Arnau, 1987) and in dry-cured Iberian ham *Pseudomonas fluorescens* were found (Andrade et al., 2012). However, in chilled meat black spots due to the growth of several species of moulds including *Cladosporium cladosporioides*, *Cladosporium herbarum*, *Penicillium hirsutum* and *Aureobasidium pullulans* have been described (Gill, Di Menna, & Lowry, 1981). Therefore, although several micro-organisms have been reported as responsible for black spot spoilage in meat products, in dry-cured fermented sausages micro-organisms involved in this spoilage still remain unclear. The growth of moulds over black spots on dry-cured fermented sausages suggests that in this product the alteration has a fungal origin.

The aim of this work was to identify the moulds responsible for black spot spoilage on dry-cured fermented sausages. For an accurate characterisation of this type of spoilage, those moulds isolated from the black spots were used to reproduce the above alteration on dry-cured fermented sausages. In addition, an accurate and rapid real-time PCR (qPCR) method to detect and quantify moulds involved in this alteration was developed.

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2. Materials and methods

2.1. Sampling and mould isolation

Samples were taken from dry-cured fermented sausages produced by a major company showing black spots. Ten grammes of sample size (1–4 mm depth) of the black spots from spoiled sausages was collected aseptically and homogenised in a Stomacher lab-blender during 1 min with 90 mL of sterile peptone water (0.1%, w/v) at room temperature. For an appropriate mould isolation, ten-fold serial dilutions were carried out with the same diluent and 0.1 mL was spread onto the surface of Potato Dextrose Agar (PDA, Scharlau Chemie S.A., Spain), Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1% peptone, 2% agar) and Dichloran Glycerol 18% Agar (DG18, Oxoid, Basingstoke, UK). Plates were incubated at two different temperatures (25 °C and 30 °C) for 4 days. About 20% of the colonies were randomly selected and subcultured (Ordóñez, 1979) according to morphological features including those isolates able to produce blackening in the former culture media. Successive isolation steps depended on picking a sample of hyphae or spores and placing this sample on a fresh PDA, MEA and DG18 plate as a point inoculum. Purity was subsequently judged by uniformity in appearance of the colony and pure colonies obtained were inoculated onto a slant of PDA and incubated at 25 °C for 4 days until being ready for identification. Spore dilution of each isolated mould was stored at −80 °C in glycerol solution (10%, w/v). The selected isolates were routinely cultured on the same medium on which they had been isolated for further assays.

2.2. Morphological strain identification

Isolated moulds were initially examined by cellular morphology under a microscope. Each suspicious blackening-producing isolate was grown on Czapek Yeast Extract Agar (CYA), MEA and Glycerol 25% Nitrate Agar (GNA) from Oxoid for 7 days at 25 °C, and also on CYA at 5 °C and 37 °C (Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996). They were tentatively characterised by morphological characteristics according to Pitt and Hocking (2009) and Núñez et al. (1996) as *Penicillium* spp. In addition, from the different isolates, only one different strain that produced blackening in the culture media was further tentatively characterised by the former morphological characterisation such as *Cladosporium* spp. This strain was named as *Cladosporium* BPS (Blackening Producing Strain).

2.3. Molecular identification of Blackening Producing Strain Cladosporium BPS

The strain BPS, that produced blackening in the culture media, was further characterised by sequencing using *β-tubulin* gene and ITS1-5.8S-ITS2 region as targets.

2.3.1. DNA extraction

Cladosporium BPS was inoculated by three-points on MEA and incubated at 25 °C for 4 days. Grown mycelium was scraped off the agar and about 50 mg of isolated mycelium was used for genomic DNA extraction following the method described by Sánchez, Rodríguez, Casado, Martín, and Córdoba (2008). DNA concentration was quantified spectrophotometrically with a Biophotometer Eppendorf (Eppendorf AG, Germany). Purified DNA was dissolved in 50 μL of sterile ultrapure water and stored at −20 °C until use for PCR reactions.

2.3.2. Sequencing of β-tubulin and ITS1-5.8S-ITS2 rRNA genes by PCR

β-Tubulin and ITS1-5.8S-ITS2 rRNA gene sequences of *Cladosporium* BPS were amplified by PCR. PCR targeted against *β-tubulin* housekeeping gene was performed using Bt2a and Bt2b primers (Glass & Donaldson, 1995). The amplification program used was: 1 cycle of 5 min at 94 °C, 32 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C and finally 1 cycle of 5 min at 72 °C. PCR reaction to amplify the ITS1-5.8S-ITS2 region was performed using ITS1 and ITS4 primers (White, Bruns, Lee, & Taylor, 1990). The amplification program used was: 1 cycle of 5 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C and finally 1 cycle of 2 min at 72 °C. After amplification, PCR products along with a DNA molecular size marker of 2.1–0.15 kbp (Roche Farma, S.A., USA) were detected on 1% (w/v) agarose gels stained with ethidium bromide (0.5 μg/mL) and visualized under an UV transilluminator. Amplification products were then purified using the MinElute® PCR Purification Kit according to the manufacturer's recommendations (QIAGEN, Hilden, Germany) and sequenced with the same primers used in the amplification steps. To avoid the amplification of artefact products, sequencing was performed from both the 5′ and the 3′ ends of each PCR product. Two sense and antisense strand sequences were edited and assembled into a consensus sequence of corresponding amplicon.

2.3.3. Sequence analysis

To determine the closest known relatives of the obtained *β-tubulin* and the ITS1-5.8S-ITS2 partial sequences, searches were performed on the GenBank database with the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Both sequences were analysed separately and 97% similarity was used as the criterion for species identification. Phylogenetic analysis of data was performed using Bayesian inference (BI) with MrBayes version 3.2.1 software. The best nucleotide substitution model was estimated with jModeltest 2.1.3, as general time reversible with estimates of invariant sites and gamma-distribution among-site rate variation. The analysis was rooted, treating *Passalora fulva* as out-group. Nodal support was estimated by posterior probabilities using the sumt command.

2.4. Develop of a specific real time PCR to quantify Cladosporium BPS

For a rapid and easy detection of the blackening-producing strain *Cladosporium* BPS, a qPCR has been optimised by using the SYBR Green methodology. To develop this method a specific pair of primers, BPS-F (5′CAACGAGGTGTGAAAATCCGA3′) and BPS-R (5′AGGCCTGTGA TGGGATGTGA3′), was designed on the basis of partial sequence alignments of the *β-tubulin* gene of various mould species usually present in dry-cured fermented sausages (Asefa et al., 2010) deposited at the National Center for Biotechnical Information (NCBI) and the sequenced *β-tubulin* partial region of the strain BPS (GenBank accession numbers: AY496000, AY495999, AF603238, JF909956, FJ004438, AY674323, JX241680, JX535302, AY819975, AY819976, AY674319, AY674317, JF521538, FJ004434, JX545088, JN394586, HQ285589, HM803081, JF521510, AY674367, JQ217372 and AY371601.1). Sequences were edited and aligned by the ClustalW2 program (www.ebi.ac.uk>/tools/msa/clustalW2). Alignment showed nonconserved regions between the *Cladosporium* BPS *β-tubulin* partial sequence and the remaining ones which were selected to design the primer pair using the Primer Express software (Applied Biosystems, Foster City, USA).

This qPCR protocol was carried out in a final volume of 12.5 μL, containing 2.5 μL of template DNA, 6.25 μL of 2× SYBR Premix Ex Taq™ (Takara Bio Inc., Japan), 0.1 μL of 50x ROX Reference Dye (Takara Bio Inc.) and 600 nM of both BPS-F and BPS-R primers. The amplification program used was: 1 cycle of 2 min at 50 °C, 1 cycle of 10 min at 95 °C and 40 cycles of 95 °C for 15 s and 70 °C for 1 min. After the final PCR cycle, melting curve analysis of the PCR products was performed by heating to 60–95 °C and continuous measurement of the fluorescence to verify the PCR product. Threshold cycle (C_t) values represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred for each amplification plot.

The specificity of primer pair was tested on genomic DNA from selected species of *Penicillium*, *Aspergillus* and *Emericella* which usually are present in dry-cured fermented sausages (Table 1). To evaluate the specificity of the designed primers for the SYBR Green assay, the