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Identification and control of moulds responsible for black spot spoilage in dry-cured ham

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ABSTRACT

The aims of this work were to identify moulds responsible for black spot spoilage in the drying and cellar stages of dry-cured ham processing and evaluate the effectiveness of preventive actions for controlling this alteration. Four mould strains isolated from spoiled hams were identified by morphological characteristics and the ITS and *β-tubulin* sequencing. Two of them were *Cladosporium oxysporum*, one was *C. cladosporioides* and the remaining one was *C. herbarum*. These spoiling strains reproduced the black spots on dry-cured ham-based media and ham slices. Additionally, the effect of water activity (a_w) conditions reached throughout dry-cured ham ripening and the activity of the protective culture *Penicillium chrysogenum* CECT 20922 against the spoiling moulds were evaluated. In the dry-cured ham model system the growth of the *Cladosporium* strains was minimised when the a_w approaches 0.84 or in *P. chrysogenum* CECT 20922 inoculated dry-cured ham slices. Therefore such combination could be used to avoid the black spot formation in dry-cured ham.

1. Introduction

Dry-cured ham is a traditional meat product obtained after several months of ripening (Toldrá, 2006). The ripening process of dry-cured Iberian ham consists of three fundamental stages: dry-salting, post-salting and drying/cellar stages. The water activity (a_w) of the surface of hams changes during processing from 0.98 a_w (at the beginning of processing) to 0.80 a_w or even lower at the end of the maturation process (Andrés, Ventanas, Ventanas, Cava, & Ruiz, 2005; Rodríguez et al., 1994). At the end of ripening, dry-cured ham usually presents pH values ranging from 5.6 to 6.5 (Rodríguez et al., 1994). These physico-chemical characteristics and the environmental conditions throughout the long ripening may favour growth of an abundant and uncontrolled mould population on the surface of hams (Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996; Peintner, Geiger, & Pöder, 2000). Despite the fact that most of these moulds are considered beneficial for the flavour development (Martín, Córdoba, Núñez, Benito, & Asensio, 2004), some of them could produce spoilage, such as the formation of black spots on the surface of the hams during drying and cellar stages. The spoiled area is superficially localised on the product provoking a depreciation of dry-cured ham when commercialised as entire pieces since consumers may not accept them. Black spot spoilage may thus lead to important economic losses for dry-cured ham manufacturing industries. A similar alteration consisting of the appearance of black spots in dry-cured ham throughout salting or post-salting stages has been reported with bacteria (*Carnimonas nigrificans*, *Pseudomonas fluorescens*) as the causative agents (Andrade, Rodas, Durbán, Moya, & Córdoba, 2012; Garriga, Ehrmann, Arnau, Hugas, & Vogel, 1998). However, in drying and cellar phases, moulds seem to be the most probable microorganisms involved in this alteration, since environmental conditions can modulate the microbial population growth. The presence of the mould population has an effect on the decrease of a_w of dry-cured ham surface. Therefore, mould population in environment is important. In addition, moulds belonging to the *Cladosporium* genus have been recently reported as responsible for black spots in other types of cured meat products such as dry-cured fermented sausages (Lozano-Ojalvo et al., 2015). Black spot spoilage has been also described in chilled meat due to the growth of several mould species including *Cladosporium cladosporioides, Cladosporium herbarum, Penicillium hirsutum* and *Aureobasidium pullulans* (Gill, Di Menna, & Lowry, 1981). Regarding dry-cured ham the etiology of black spots found in drying and cellar stages still remains unclear.

Furthermore, it should be necessary to take measures to control this alteration. Thus, preventive actions that rely on the a_w reduction that occurs throughout the dry-ripening process and/or the use of protective cultures could be considered. The usefulness of non-toxigenic moulds as antagonistic towards unwanted moulds commonly found in dry-cured meat products has been demonstrated (Bernáldez et al., 2013; Rodríguez et al., 2015). Concretely, the strain *P. chrysogenum* CECT 20922 (formerly *P. chrysogenum* RP42C) has shown a strong antifungal

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activity attributed to the PgAFP protein (Rodríguez-Martín et al., 2010). Such protein has revealed effects against the main toxigenic mould species colonising dry-cured meat products when tested on culture medium and dry-fermented sausages (Delgado et al., 2015). Given that this strain has demonstrated high ability to grow in the conditions associated with ripening of dry-cured meat products (Acosta, Rodríguez-Martín, Martín, Núñez, & Asensio, 2009), its use could be evaluated to avoid the growth of moulds involved in black spot formation.

The aim of this work was to identify the moulds responsible for black spot spoilage in dry-cured ham during the drying and cellar stages. Additionally, the effect of the a_w reduction and/or the use of the strain *P. chrysogenum* CECT 20922 on controlling moulds causing the black spots were evaluated.

2. Materials and methods

2.1. Sampling and mould isolation

Samples were taken from dry-cured hams showing black spots in the drying and cellar stages in two large meat processing plants (Fig. 1). Ham surfaces of 25 cm^2 containing black spots (Fig. 1a) were completely scraped by a sterilised steel scalpel, transferred to sterile plastic bags and quickly transported to the laboratory at 4 °C. Then, samples were homogenised with 90 mL of 0.1% sterile peptone water (w/v) in a Stomacher lab-blender for 1 min at room temperature. Tenfold serial dilutions were then made with the same diluent and 0.1 mL was spread onto Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1% peptone, 2% agar). After incubating the plates at 25 °C for 5 days, about 20% of the mould colonies were randomly selected and subcultured (Ordóñez, 1979) according to morphological features including those colonies producing blackening in the former culture medium. From each MEA plate, the colonies of the potentially spoilage moulds were selected for further characterisation. The obtained pure mould colonies were inoculated on Potato Dextrose Agar (PDA; Scharlau Chemie S.A., Spain) and MEA and incubated at 25 °C for 7 days. Spores of each isolated mould were harvested by flooding 3 plates of MEA and PDA with 5 mL of sterile nanopure water containing 10% glycerol (v/v; Scharlau Chemie S.A.) and gently rubbing the surface with a glass rod to remove the spores. The spore suspensions were counted using a Thoma counting chamber and stored at −80 °C until required.

2.2. Morphological identification of blackening-producing mould isolates

The mould isolates able to produce blackening in the culture media were initially examined by cell morphology under a microscope Eclipse E200 equipped with a digital camera DS-Fi2 (Nikon, Japan). Each blackening-producing mould isolate was then tentatively identified by growing on Czapek Yeast Extract Agar (CYA), MEA and Glycerol 25% Nitrate Agar (G25 N) for 7 days at 25 °C, and on CYA at 5 °C and 37 °C (Pitt & Hocking, 2009). All assays were done in triplicate. A total of four different mould isolates able to produce blackening in the former culture media were obtained. They were named as N3, N4, N4b and N5. Such isolates were maintained by regular subculturing on PDA or MEA at 25 °C for 7 days and then stored at 4 °C.

2.3. Molecular identification of blackening-producing isolates

The four mould isolates able to produce blackening in the culture media were further identified by sequencing of the ITS1-5.8S-ITS2 region and *β-tubulin* gene.

2.3.1. DNA extraction

One hundred μL of a spore suspension of about 6 log spores/mL of each isolate were inoculated on Malt Extract Broth (MEC) and incubated by shaking for 4 days at 25 °C and 200 rpm. The obtained mycelium was

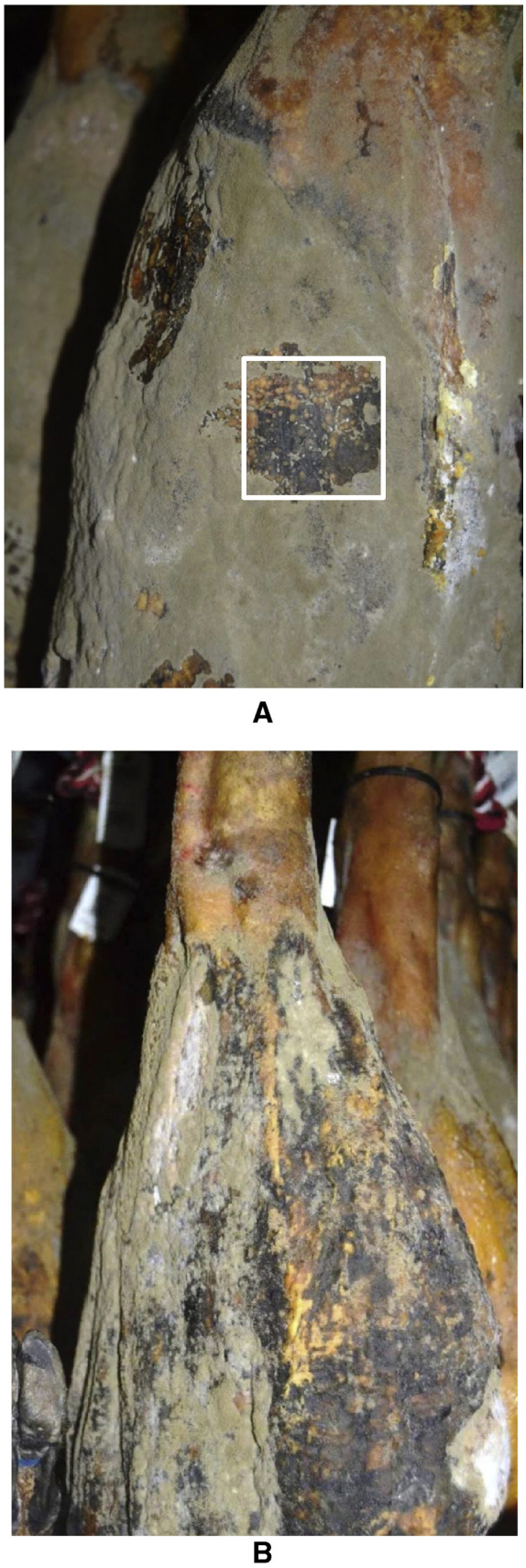

Fig. 1. Dry-cured ham showing black spot spoilage during drying and cellar stages. a) Ham surface scraped off by a sterilised steel scalpel for sampling; b) surface of dry-cured ham with black spots covering the whole surface.