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Influence of ochratoxin A on adaptation of *Penicillium nordicum* on a NaCl-rich dry-cured ham-based medium

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ABSTRACT

Iberian dry-cured ham is an important meat product with high consumption worldwide. The special ecological conditions occurring throughout its ripening favour surface colonisation of filamentous fungi. Normally, moulds contribute to the development of the sensory qualities of the ham; however, some toxigenic species, such as *Penicillium nordicum*, are able to successfully adapt to the NaCl-rich environment found in dry-cured ham and produce ochratoxin A (OTA) in this product. Moreover, it was suggested that the biosynthesis of OTA by *P. nordicum* itself may support the adaptation to this food environment. However, this mechanism has not been completely elucidated yet. The objective of this work was to evaluate the influence of different concentrations of commercial OTA (cOTA, at 0, 0.2, 1 and 5 ppb) on growth rate, biosynthetic- and stress-related gene expression and OTA production by two *P. nordicum* strains (Pn15 and Pn69) on dry-cured ham based-media. Two NaCl conditions (0% and 10%) were evaluated for each cOTA level. In general, no intra-strain and inter-strain differences in growth rates were found among the conditions tested. The stress-related *Hog1* gene expression of the strain Pn15 was affected by cOTA and NaCl concentration whilst the strain Pn69 was not affected by these variables. The expression of OTA-related *otapks* and *otanps* genes of the strain Pn15 was affected by several NaCl and cOTA combinations. However, the strain Pn69 showed no differences in relative gene expression. Regarding to OTA production, different behaviours were displayed by the two strains. The strain Pn15, which produced high OTA amounts by itself, produced OTA without the necessity of the presence of NaCl or cOTA as stressors. However, the presence of cOTA triggers OTA production by the weak OTA producing Pn69 in the absence of NaCl. In addition, although a moderate correlation was found between the expression of the OTA-related genes and mycotoxin produced by *P. nordicum* in the absence of NaCl, none was obtained between *Hog1* gene expression and mycotoxin production. This study is a step forward for a better understanding of the ability of *P. nordicum* producers of OTA to colonise NaCl-rich habitats such as Iberian ham for proposing actions to minimise OTA contamination in this meat product.

1. Introduction

Dry-cured ham is an important and typical meat product with high consumption in many countries, especially those placed in the Mediterranean area such as Spain, Portugal and Italy (Toldrá and Aristoy, 2010). Many types of dry-cured hams are produced throughout the world (Asefa et al., 2009; Laureati et al., 2014; Théron et al., 2010). Among them, Iberian hams are economically important foods produced under regulation of different Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) such as “Barrancos”, “Dehesa de Extremadura” or “Guijuelo” and consumed in Europe and exported world-wide.

The processing of Iberian hams consists in three stages: dry-salting, post-salting and ripening/ageing. The Iberian ham has a unique and distinguishing salting stage since hams are salted in piles with alternate beds of hams and NaCl at low temperatures (< 5 °C) (Martín et al., 1998). Then, diffusion of salt (NaCl) into the inner muscles of the hams results in a reduction of the water activity (a_w), over 2–3 additional months in the post-salting stage at low temperatures (< 5 °C). After post-salting stage, hams are placed at maturation chambers, where temperatures are progressively increased from 7 to 10 °C to 25–30 °C. Ripening time depends on the weight and characteristics of product,

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but, in general, sensory quality of ham improves as long as this period is (18–24 months). Because of this long ripening time, the final NaCl concentration varies between 10 and 20% in dry matter (Arnau et al., 1995) and the a_w values may vary between 0.93 and 0.84 a_w depending on the product and the length and conditions of the processing (Andrés et al., 2005). This a_w decrease ensures the microbial stability and safety of this meat product. The special ecological conditions occurring throughout the ripening, especially at the end of this stage when the temperature is higher, favour surface colonisation of filamentous fungi, which can contribute to enhance the flavour development (Martín et al., 2006). However, some toxigenic mould species, such as *Penicillium nordicum*, *Penicillium verrucosum* or *Aspergillus westerdijkiae* are able to successfully adapt to the characteristic NaCl-rich environment of the dry-cured meat products (Rodríguez et al., 2014; Schmidt-Heydt et al., 2012; Vipotnik et al., 2017). Therefore, their ability to produce mycotoxins in the products has been studied, being ochratoxin A (OTA) the mycotoxin most frequently detected (Markov et al., 2013; Pleadin et al., 2015; Rodríguez et al., 2012; Toscani et al., 2007). This metabolite is considered genotoxic due to formation of OTA-DNA adducts (Ostry et al., 2016). It has also shown nephrotoxic, hepatotoxic and immunotoxic properties (Schmidt-Heydt et al., 2011). In addition, this mycotoxin has been classified by the International Agency for Research on Cancer as Group 2B carcinogen (possibly carcinogenic to humans) (Ostry et al., 2016). So far, only Italian government has set legal limits for this toxin of 1 μg/kg OTA in dry-cured ham (Ministerio della Sanità, 1999).

In particular, *P. nordicum* has demonstrated to be an important and consistent producer of this mycotoxin in NaCl-rich products such as dry-cured ham (Rodríguez et al., 2014, 2015; Sonjak et al., 2011). The high content of NaCl in these products, which provokes osmotic stress, affects adaptation of this specific fungal population. Particularly, OTA production by *P. nordicum* has been hypothesised to be a key factor in the maintenance of chloride homeostasis. The production of this mycotoxin, which contains a chloride atom, and its subsequent excretion would contribute to ensure the chloride homeostasis within the fungal cell (Schmidt-Heydt et al., 2011). However, this mechanism has not been completely elucidated yet. Thus, further studies are required to fully understand the advantageous adaptation of *P. nordicum* and its ability to produce OTA in dry-cured meat products. In addition, OTA accumulation may modulate intracellular OTA production. Therefore, a deeper knowledge on *P. nordicum* physiology related to the constituents of the meat matrix, and the presence of the mycotoxin itself with regard to OTA production would allow designing new strategies to prevent OTA accumulation on Iberian ham and achieve the objective to manufacture dry-cured ham free of OTA.

The aim of this work was to evaluate the influence of exogenous OTA and NaCl on growth rate, mycotoxin biosynthetic- and stress-related gene expression and OTA production of two *P. nordicum* strains grown on dry-cured ham based-media.

2. Materials and methods

2.1. Mould strains and culture conditions

Two OTA-producing *P. nordicum* strains were evaluated in this work. One of them was obtained from the Centraalbureau voor Schimmelcultures (CBS) fungal collection (Utrecht, The Netherlands), *P. nordicum* 110.769 (Pn69), and used as reference strain. The other one, *P. nordicum* 15 (Pn15), was isolated from dry-cured ham and held in the Culture Collection of the Food Hygiene and Safety Group at the University of Extremadura (Cáceres, Spain). Both strains were routinely grown on Potato Dextrose Agar (PDA; Scharlab S.L., Spain) for 7 days at 25 °C. Spores from surface of agar plates were collected using 5 mL of phosphate saline buffer (PBS) and rubbing the surface with a sterile glass rod. The spore suspensions were maintained in 10% glycerol solutions at −80 °C and new cultures were used for each experiment.

2.2. Culture media preparation

Dry-cured ham was lyophilised for 24 h at 5 °C in Bulk Tray Dryer with 6-Port Manifold coupled to FreeZone 6 Liter Console Freeze Dry System (Labconco, USA). Dry-cured ham-based agar was prepared by mixing 30 g of lyophilised dry-cured ham, 20 g of Bacto agar (Scharlab S.L.) and 1000 mL of deionised water (0.984 a_w). Additionally, the basic medium was supplemented with 100 g/L of NaCl (0.934 a_w) to reach the similar salt concentration to that found in an Iberian dry-cured ham at the ripening stage. The culture media were prepared by autoclaving for 20 min at 121 °C. After that, commercial OTA (cOTA; Sigma-Aldrich, Spain) was added to both supplemented and non-supplemented media NaCl media to obtain different final concentrations of this mycotoxin (0.2, 1 and 5 ng cOTA/g of medium). Also, non-cOTA added media were used for the experiments (control). Flasks of molten media were vigorously shaken and pouring into 55-mm diameter sterile Petri plates. The a_w values for media were measured in the water activity meter Novasina Lab Master from Novasina AG (Switzerland), being 0.984 and 0.934 a_w for non-NaCl and NaCl supplemented media, respectively. The treatments were enclosed in separate polyethylene bags to maintain constant the a_w level during the experiment.

2.3. Inoculum preparation, inoculation and experimental conditions

Spore suspensions from *P. nordicum* strains were taken as described in Section 2.1. They were counted using a Thoma chamber and adjusted to 10^6 spores/mL by diluting with PBS and used as inoculum.

For growth and OTA production assays, agar plates were centrally inoculated with 2 μL of the inoculum. For gene expression assays, sterile cellophane overlays (Packaging Limited, UK) were placed onto media before inoculation.

The agar plates were incubated at 25 °C for up to 14 days. All experiments were performed with three replicates per treatment.

2.4. Growth rate calculations

Radial growth was daily recorded by measuring two right-angled diameters. Data were analysed using a primary model by plotting colony diameter against time. Data plots showed, after a lag phase, a linear trend with time. The linear part of this graph (linear phase) was used to calculate growth rate (μ, mm/d) (Garcia et al., 2009).

2.5. Gene expression studies

2.5.1. Sampling and sample preparation

For gene expression studies, sampling was performed at 7 days of incubation. This time frame was chosen because previous studies with *P. nordicum* suggested that gene expression of the two biosynthetic genes was optimal after 6–7 days of incubation (Bernáldez et al., 2017; Rodríguez et al., 2014). Cellophane sheets containing whole colonies were collected under sterile conditions, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.5.2. RNA extraction

Frozen mycelia were ground to fine powder in a pre-frozen mortar and pestle. RNA extraction was carried out by using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) following manufacturer's instructions (Protocol A). The RNA concentration (μg/μL) and purity (A_{260}/A_{280} ratio) were spectrophotometrically determined using a 1.5 μL aliquot on a NanoDrop™ (Thermo Fisher Scientific, USA). Samples were diluted to 0.1 μg/μL and treated with DNAse I kit (Thermo Fisher Scientific, USA) to remove genomic DNA traces as described by manufacturer.

2.5.3. Two steps reverse transcription quantitative PCR

RT-qPCR assays were used to amplify the key genes involved in OTA