International Journal of Food Microbiology 272 (2018) 22–28

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

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

Josué Delgado, Lucía da Cruz Cabral[1], Mar Rodríguez, Alicia Rodríguez*

Food Hygiene and Safety, Meat and Meat Products Research Institute, Faculty of Veterinary Science, University of Extremadura, Avda. de las Ciencias, s/n. 10003 Cáceres, Spain

ARTICLE INFO

Keywords:
Penicillium nordicum
Iberian ham
Ochratoxin A
NaCl
Stress

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,

* Corresponding author.
E-mail address: aliciarj@unex.es (A. Rodríguez).
[1] Present address: Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica, Laboratorio de Microbiología de Alimentos, Buenos Aires, Argentina.

https://doi.org/10.1016/j.ijfoodmicro.2018.02.020
Received 14 October 2017; Received in revised form 17 January 2018; Accepted 17 February 2018
Available online 21 February 2018

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

Table 1
Nucleotide sequences of primers and primers´ concentrations for RT-qPCR assays used in this work.

Primer	Gene	Nucleotide sequence (5′–3′)	Reference	Primer concentration (nM)
F-npstr	*otanps*	GCCGCCCTCTGTCATTCCAAG	Rodríguez et al. (2011)	400
R-npstr		GCCATCTCCAAACTCAAGCGTG		400
otapksF3	*otapks*	CGCCGCTGCGGTTACT	Bernáldez et al. (2017)	300
otapksR3		GGTAACAATCAACGCTCCCTCTT		300
HogF2	*Hog1*	GGTAGACATCTGGAGCGCGG	Rodríguez et al. (2016)	150
HogR2		TCACATCATCGGGAGGAGTA		150
β-tub F1	*β-tubulin*	GCCAGCGGTGACAAGTACGT	Bernáldez et al. (2017)	300
β-tub R1		TACCGGGCTCCAAATCGA		300

biosynthetic pathway, *otapks* and *otanps* genes and the osmotic stress-related gene, *Hog1*. The *β-tubulin* gene was used as endogenous control.

2.5.3.1. cDNA synthesis. Five μL of total RNA (500 ng) were used for reverse transcription reaction using PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) as described by the manufacturer. cDNA samples were stored at −20 °C until analysis.

2.5.3.2. qPCR reactions. The qPCR reactions were performed in the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Life Technologies, USA) using the SYBR Green technology. Primer sets, mix reactions and thermal conditions of the qPCR reactions were essentially the same that those used previously (Bernáldez et al., 2017; Rodríguez et al., 2014, 2016). The nucleotide sequences of the primers and concentrations used are shown in Table 1. Quantification cycle (Cq) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated by the ViiA™ 7 software version 1.2.4. (Applied Biosystems).

2.5.3.3. Relative quantification of the gene expression. Relative quantification of the expression of the OTA-related genes (*otapks* and *otanps*) and stress-related gene (*Hog1*) was calculated according to the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001), after checking that requirements needed to use it were met. The *β-tubulin* gene was used as endogenous control to normalise the quantification of the mRNA target for avoiding errors caused by the multistage process required to extract, process and detect mRNA. This method allows calculation of the expression ratio of a target gene between a tested sample and its relative calibrator (control sample). In this work, the calibrator corresponded to *P. nordicum* when grown on non-modified media (neither NaCl nor cOTA added).

2.6. Extraction and quantification of OTA

2.6.1. Sampling and sample preparation

For determining OTA production, samples were collected by day 14 of incubation. Then, inoculated and incubated dry-cured ham-based media weighing around 2 g were placed into 50 mL conic tube. All replicates per treatment were immediately frozen at −20 °C and stored until use.

2.6.2. OTA extraction procedure

The OTA extraction was based on the QuEChERS procedure described by Kamala et al. (2015). Briefly, this method included extraction with water and acetonitrile (Scharlab S.L.) acidified with acetic acid 0.1% (v/v; Fisher Scientific, USA) prior to phase partitioning using NaCl (Scharlab S.L.) and anhydrous $MgSO_4$ (Scharlab S.L.). The mixture was immediately and vigorously shaken by hand and centrifuged for 5 min at 4 °C at 5300 rpm (Diglicen 21R, Ortoalresa, Spain). After that, 0.75 mL aliquot from the supernatants were evaporated. Dry extracts were then redissolved in 0.75 mL HPLC-grade methanol (Scharlab S.L.), filtered through a 0.22 μm pore size nylon membrane (Jet Bio-Filtration Co., Ltd., China) and stored at −20 °C until analysis.

2.6.3. Quantification of OTA by HPLC-FLD

OTA quantification was performed in an Agilent 1260 Infinity (Agilent Technologies, USA) equipment coupled to a fluorescence (FLD) detector (Agilent Technologies, UK) applying the method reported by Rodríguez et al. (2014). A Phenomenex C_{18} column of 250 × 4.6 mm, 5-μm particle size (Phenomenex, USA) was used. The mobile phase was water:acetonitrile:acetic acid (41:57:2 v/v/v) with a flow rate of 1.0 mL/min. Injection volume was set in 20 μL. FLD detection was performed using 333 nm and 460 nm excitation and emission wavelengths, respectively. The run time for samples was 15 min and the retention time for OTA was 7.5 min. The full FLD spectra data were acquired and processed using the Agilent Technologies software (Agilent, USA).

Standard solutions of OTA were used (from 1 to 100 ng/mL) to build a calibration curve by HPLC-FLD. This curve revealed a linear relationship (R^2 = 0.998) between detector response and amount of OTA standard (Sigma Aldrich Química S.A., Spain). Limits of detection and quantification (LOD and LOQ) were determined as previously described (Long and Winefordner, 1983) and were 1.06 μg/kg and 3.24 μg/kg, respectively.

When cOTA was added to the media, its concentration was subtracted from total OTA quantified.

2.7. Statistical analysis

Statistical analyses on growth rate, relative gene expression and OTA concentration datasets were performed using the IBM SPSS v.22 (www-03.ibm.com/software/products/es/spss-stats-standard). Data sets were tested for normality (Kolmogorov–Smirnov with Lilliefors correction) and homoscedasticity (Levene's test). The whole sets of results were not normally and homoscedastic distributed, thus median values were compared using the non-parametric Kruskal–Wallis test. To compare treatments in pairs, Mann–Whitney U test was applied. The statistical significance was set at $p \leq 0.05$. Moreover, single correlation analysis between OTA level and gene expression was calculated in pairs using Spearman's rank correlation coefficients.

3. Results

3.1. Mould growth

The influence of different concentrations of cOTA on *P. nordicum* growth rate inoculated on modified and non-modified medium with NaCl is shown in Fig. 1. The growth rates observed were between 0.81 and 1.42 mm/day or 0.89 and 1.22 mm/day for the strains Pn15 and Pn69, respectively. In general, growth rates of the two strains were not affected by any tested cOTA concentration regardless of the presence of NaCl in the media.

3.2. Relative gene expression

3.2.1. Stress-related gene expression

The relative expression of the *Hog1* gene related to osmotic stress by

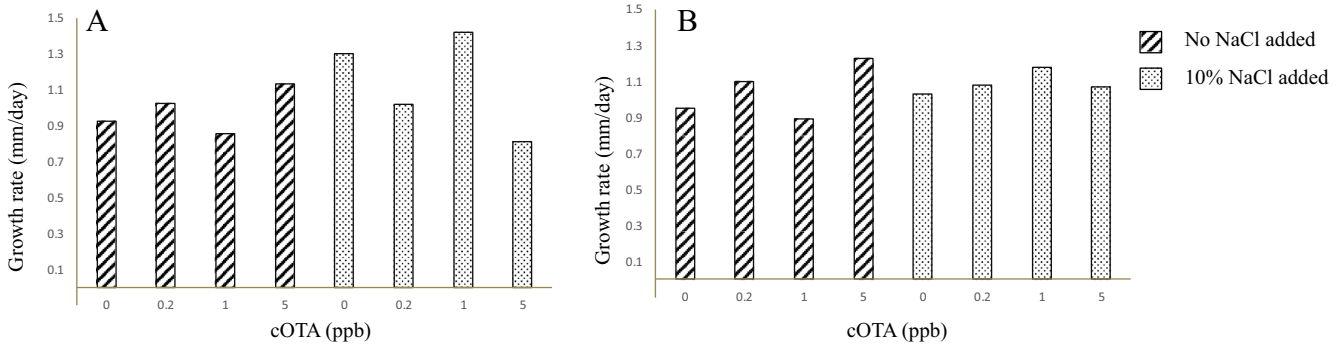

Fig. 1. Growth rates of Pn15 (A) and Pn69 (B) inoculated on dry-cured ham-based media with (10%) and without NaCl in combination with 0, 0.2, 1 and 5 ppb of cOTA and incubated for 7 days at 25 °C. *Denotes differences ($p \leq 0.05$) between non-supplemented samples neither with cOTA nor NaCl and the remaining samples.

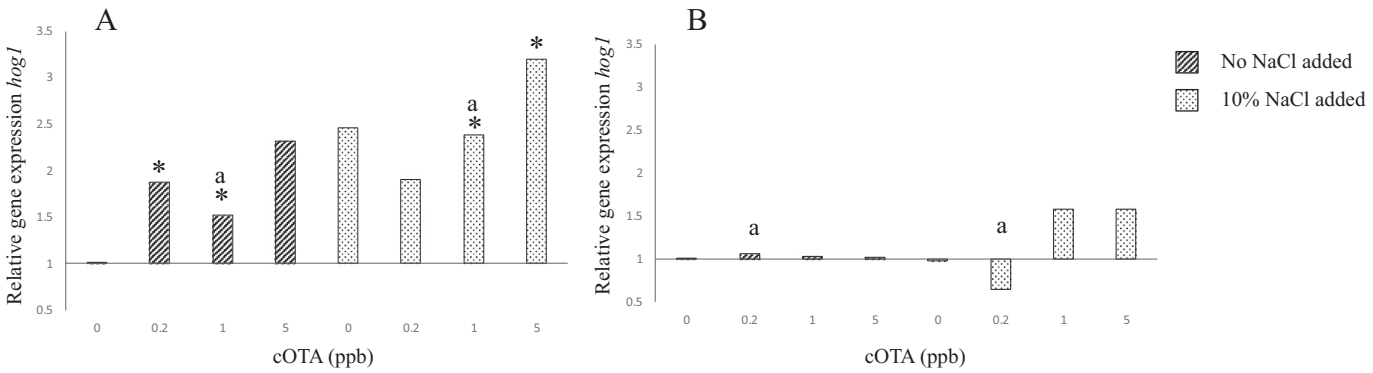

Fig. 2. *Hog1* relative gene expression of Pn15 (A) and Pn69 (B) inoculated on dry-cured ham-based media with (10%) and without NaCl added in combination with 0, 0.2, 1 and 5 ppb of cOTA and incubated for 7 days at 25 °C. *Denotes differences ($p \leq 0.05$) between samples and the calibrator. [a]Means differences ($p \leq 0.05$) between samples in the absence and presence (10%) of NaCl at the same quantity of cOTA added.

both *P. nordicum* strains is shown in Fig. 2. The influence of both NaCl and cOTA on the relative expression of this stress-related gene was evaluated and compared with the control samples when *P. nordicum* strains were grown on a non-modified ham-based medium (calibrator). For the strain Pn15 (Fig. 2A), all conditions evaluated showed the trend of an overexpression of the *Hog1* gene. Interestingly, in NaCl-supplemented agar plates inoculated with the strain Pn15, the highest levels of cOTA (1 and 5 ppb) provoked significant increments in the expression of this stress-related gene. This was also observed in non-NaCl supplemented medium at all cOTA amounts added.

Regarding strain Pn69, there were not significant differences in the transcription levels of the *Hog1* gene with respect to the calibrator (Fig. 2B).

3.2.2. OTA-related gene expression

The relative expression of the *otapks* and *otanps* genes of the two strains of *P. nordicum* is displayed in Fig. 3. The effect of both NaCl and cOTA on the relative expression of the two biosynthetic genes was tested and compared with the control samples when *P. nordicum* strains were grown on a non-modified ham-based medium (calibrator). In general, comparing expression levels of both OTA biosynthetic genes (*otapks* and *otanps*) of the two strains, the *otapks* gene expression levels were higher than the *otanps* gene ones ($p \leq 0.05$). There were inter-strain differences in the transcription levels of both genes ($p \leq 0.05$). In addition, there were intra-strain differences in the relative expression values of the two OTA-related genes of the two strains in the conditions evaluated ($p \leq 0.05$). For the strain Pn15, a significant increase of the *otanps* gene expression was observed at 1 ppb of cOTA for NaCl and non-NaCl agar plates and at 5 ppb cOTA in NaCl-supplemented agar plates (Fig. 3A). For the *otapks* gene, higher relative expression values were found when 1 ppb of cOTA was added to non-NaCl plates, and with 5 ppb of cOTA in NaCl-plates (Fig. 3B).

For the strain Pn69, in general no significant differences were detected between transcription levels of both OTA-related genes regarding to the calibrator (Fig. 3C and D).

3.3. OTA production

The OTA production by the two *P. nordicum* strains in the different conditions tested is shown in Fig. 4. For the strain Pn15, there was a significant increase of OTA production in non-NaCl plates when 0.2 ppb of cOTA was added ($p \leq 0.05$). On the other hand, a decrease in OTA accumulation was detected when 5 ppb of cOTA was added for NaCl-modified media (Fig. 4A).

For the strain Pn69, no OTA was detected when grew on non-supplemented medium neither with NaCl nor with cOTA. In addition, this strain showed two different OTA production profiles depending on the NaCl supplementation. For non-NaCl plates, this strain produced higher amounts of OTA as higher levels of cOTA were added in the media obtaining a strong correlation between cOTA and OTA production ($p \leq 0.01$) with a ρ value = 0.957. On the contrary, in the NaCl-supplemented plates, this strain synthesised lower quantities of OTA as the cOTA amounts added to the medium increased, finding a negative correlation between these two parameters ($\rho = -0.845$; $p \leq 0.05$).

Additionally, the influence of the NaCl on OTA biosynthesis by *P. nordicum* was evaluated for each level of cOTA. In all cases, both strains produced more amounts of the mycotoxin when no salt was added to the media. The strain Pn69 produced more OTA amounts at the highest levels of cOTA (1 and 5 ppb); while for the strain Pn15 this was true at the lowest levels (0 and 0.2 ppb of cOTA) in non-NaCl plates.

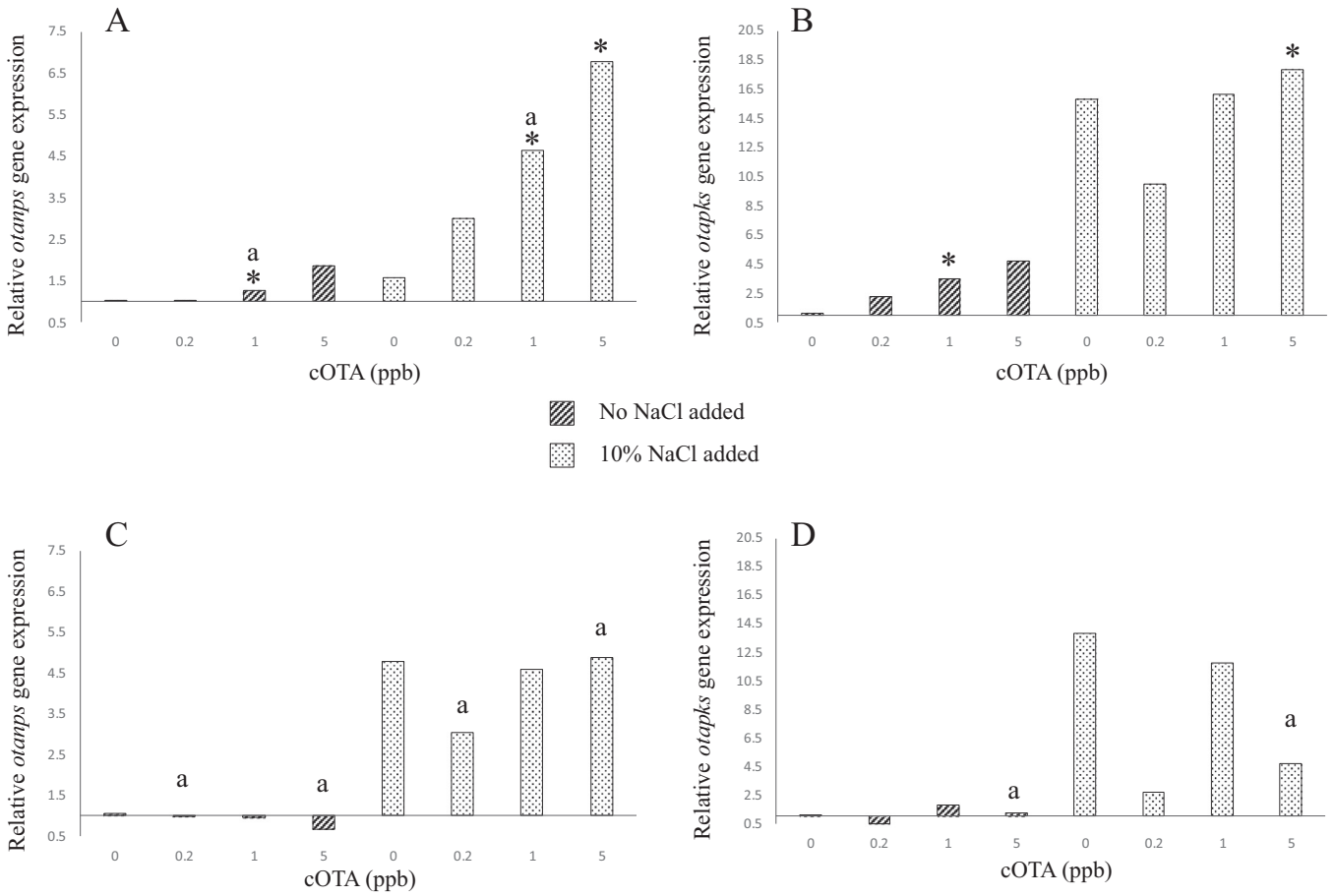

Fig. 3. Relative expression of the *otanps* (A, C) and *otapks* (B, D) genes of Pn15 (A, B) and Pn69 (C, D) inoculated on dry-cured ham-based media with (10%) and without NaCl added in combination with 0, 0.2, 1 and 5 ppb of cOTA, for 7 days at 25 °C. *Denotes differences ($p \leq 0.05$) between samples and the calibrator. [a]Means differences ($p \leq 0.05$) between samples in the absence and presence (10%) of NaCl at the same quantity of cOTA added.

3.4. Relationship between OTA production and relative expression of OTA-biosynthetic and stress-related genes

The relationship between gene expression and OTA phenotypic production was analysed by the Spearman's rank correlation test and results are shown in Table 2. The OTA production was not related with the *Hog1* gene expression ($p > 0.05$) by *P. nordicum*. A moderate correlation was found between the *otapks* and *otanps* gene expression data and OTA amounts produced by *P. nordicum* when grown on the ham-based medium without NaCl (Table 2).

4. Discussion

To the best of our knowledge, this is the first study that evaluates the influence of exogenous OTA, in combination with NaCl concentration usually encountered in Iberian dry-cured ham during its processing, on *P. nordicum* adaptation to this kind of NaCl-rich food product. OTA, which has been considered a toxic molecule for the cell (Schmidt-Heydt et al., 2011), may provoke an external stimulus (as stressor) that triggers a cellular response in *P. nordicum*. This research arose from the hypothesis that the biosynthesis of OTA itself may

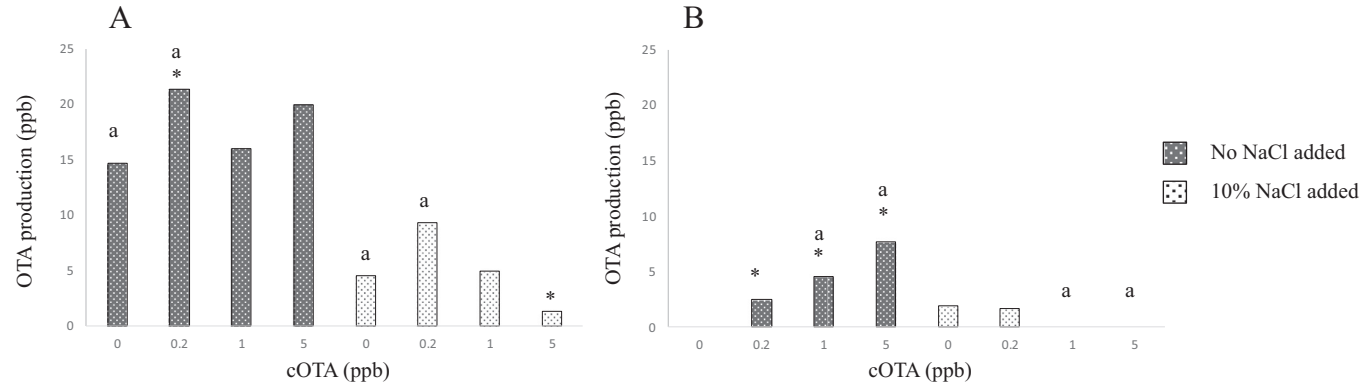

Fig. 4. OTA production by Pn15 (A) and Pn69 (B) inoculated on dry-cured ham-based media with (10%) and without NaCl in combination with 0, 0.2, 1 and 5 ppb of cOTA incubated for 7 days at 25 °C. *Denotes differences ($p \leq 0.05$) between non-supplemented samples neither with cOTA nor NaCl and the remaining samples. [a]Means differences ($p \leq 0.05$) between samples in the absence and presence (10%) of NaCl at the same quantity of cOTA added.

Table 2
Coefficients of correlation from Spearman's rank test for ochratoxin A (OTA) levels and relative gene expression of *Penicillium nordicum* when grown in dry-cured ham-based media in the presence and absence of NaCl.

Gene	Dry-cured ham medium	ρ-value	*p* value
Hog1	Without NaCl	0.427	0.060
	With NaCl	0.127	0.603
otapks	Without NaCl	0.463	0.040
	With NaCl	0.107	0.671
otanps	Without NaCl	0.541	0.017
	With NaCl	0.399	0.101

contribute to the colonisation of *P. nordicum* to such highly specialised foods under osmotic stress where very few other moulds are able to grow (Schmidt-Heydt et al., 2012). Growth, expression of a stress-related gene and two key genes involved in OTA biosynthesis and the subsequent OTA production by two strains of *P. nordicum* were assessed. The information yielded in this work is interesting for a better understanding of the mechanism of adaptation of *P. nordicum* to this food environment to design strategies to prevent OTA contamination in ham.

The results obtained in the present study did not show significant differences between the colonisation of the two *P. nordicum* strains at the experimental conditions assayed. It seems that in general the presence of cOTA did not promote the growth of both strains in both supplemented and not supplemented culture media with NaCl. In addition, no significant effect of NaCl concentration on growth rates at the same cOTA amount was found. These results were not surprising, as this species is able to grow in a similar way in a wide range of NaCl concentrations (Rodríguez et al., 2014, 2016; Schmidt-Heydt et al., 2012).

The transcriptomic approach included molecular studies on OTA- and stress-related genes of both *P. nordicum* strains in response to the different cOTA concentrations at two NaCl conditions. The targeted genes were *otanps* and *otapks*, key genes involved in OTA biosynthesis (Karolewiez and Geisen, 2005), as well as the osmotic stress-related *Hog1* gene. The *otanps* and *otapks* genes have been previously utilised to monitor OTA production by *P. nordicum* in NaCl enriched culture media (Rodríguez et al., 2014; Schmidt-Heydt et al., 2012), while the *Hog1* gene expression was useful for studying osmotic stress changes by *P. nordicum*, given that this gene mediates adaptive responses to osmotic stress (Hayes et al., 2014; Rodríguez et al., 2016).

In this study, the strains Pn15 and Pn69 showed different profiles for the relative expression of the *Hog1* gene. In general, for the strain Pn15 the activity of the gene increased when higher cOTA amount was added to the ham-based medium regardless of NaCl concentration. However, the strain Pn69 displayed no differences in gene expression neither due to NaCl content nor cOTA added. Another *P. nordicum* strain showed higher *Hog1* transcription levels at 10% NaCl than 0% NaCl in YES medium, but this increment was similar for up to 40% NaCl tested (Rodríguez et al., 2016).

The expression profiles of the two biosynthetic genes were also quite different for both ochratoxigenic strains assayed. The *otapks* and *otanps* gene expression displayed by the strain Pn15 tended to increase at 10% NaCl and at higher cOTA concentration (Fig. 3A and B). For the strain Pn69, the relative expression patterns for these two OTA-related genes were quite similar to those observed in the same strain cultured on ham-based medium supplemented with 10% of NaCl (Rodríguez et al., 2014). In addition, the *otapks* and *otanps* gene expression levels for both strains in the absence of cOTA were not modified by the reduction of a_w values at 0.94, provoked by the addition of 10% of NaCl. This a_w value, reached with a non-ionic compound (glycerol), has been reported to trigger a peak of gene induction for *Aspergillus parasiticus*, *Fusarium culmorum* and *P. verrucosum*, linked to conditions where the growth was greatly reduced (0.93 a_w), suggesting that the activation of mycotoxin biosynthesis is stress regulated (Schmidt-Heydt et al., 2008).

OTA amounts produced by the two *P. nordicum* strains at the different experimental conditions assayed were quite different (Fig. 4). The strain Pn15 was able to produce 15 ± 0.99 ppb of OTA in ham-based media without cOTA and NaCl addition; however, no OTA was detected by the strain Pn69 at these conditions. These opposite behaviours may be explained by the global intra-species differences found with regard to OTA production, given that the strain Pn15 seems to not require any external stimuli to trigger OTA production whilst the strain Pn69 needs cOTA or NaCl as external stimulus to produce this mycotoxin.

The strain Pn15 produced OTA within a range between 15 and 23 and 2–10 ppb in the absence and presence of NaCl, respectively. Interestingly, OTA amounts produced by this strain were independent of the cOTA concentration added at both salt environments studied. On the other hand, the strain Pn69 produced higher levels of OTA on non-NaCl medium as the quantity of cOTA added increased (Fig. 4B). This response clearly points to cOTA is recognised by this strain as a kind of stressor signal. However, this signal seems to be not recognised in the presence of 10% NaCl. This fact may be explained by the stronger stimulus caused by the salt itself which would impede to identify the small amount of cOTA added as a stressor. In addition, the lack of differences ($p > 0.05$) between the OTA production by this strain when no cOTA was added in the presence and absence of NaCl are in accordance with findings reported for other *P. nordicum* strain (Schmidt-Heydt et al., 2012). However, that strain produced much higher quantities of OTA than the strain Pn69, these differences can be attributed to intra-species differences as well as differences on the matrices used.

The Spearman's rank analysis showed the relationship between the transcriptomic status of both *P. nordicum* strains and the OTA production, either in the presence or absence of NaCl (Table 2). The relationship between the *otanps* and *otapks* gene expression and OTA production was not significant in the presence of NaCl, whilst there was a correlation between both parameters in the absence of this salt. Interestingly, no relationship was found between the *Hog1* gene expression and OTA production when *P. nordicum* strains were cultured on modified or non-modified ham-based media.

It is commonly accepted that *Hog1* expression is exclusively affected by osmotic stress (Schüller et al., 1994), although some authors have suggested that the expression of this gene could be modified by other compounds. In fact, the expression of the *Hog1* gene seems to play some role in the oxidative stress response (Singh, 2000). The activation of stress-related pathways could switch pathways on linked to mycotoxin biosynthesis, as it has been reported for the biosynthesis of trichothecenes and alternariol by *Fusarium graminearum* and *Alternaria alternata*, respectively (Graf et al., 2012; Lin and Chung, 2010). Considering that OTA is toxic for the cell and seems to be immediately excreted out by the producer (Schmidt-Heydt et al., 2011), then it could be hypothesised a cellular response against exogenous OTA. Although it seems that exogenous OTA in meat products with low content of NaCl may act as stressor on weak *P. nordicum* strains producer of OTA and activate this harmful mycotoxin production, the results obtained in the present study rule out that activation of the *Hog1* gene expression is triggered by cOTA for both strains. Other stress-related pathways must be activated by the presence of exogenous OTA, so further studies are required to elucidate the action mechanism of this stressor to activate the OTA biosynthetic pathway.

5. Conclusions

The influence of interacting conditions of exogenous OTA combined with NaCl concentration usually found in Iberian ham does not have a profound effect on relative growth rates by *P. nordicum* strains, confirming that this species is well adapted at this challenging environment. However, intra-species differences have been found at transcriptomic level and on OTA production in response to NaCl and cOTA. Whether *P. nordicum* strain produces high OTA amounts by itself, this

mycotoxin is produced after activation of the stress- and biosynthetic-gene pathways without the necessity of the presence of NaCl or exogenous OTA as stressors. In the case of weak OTA producing *P. nordicum* strains, the presence of exogenous OTA triggers OTA production by this mould species in the absence of NaCl. Further studies to discern the mechanisms associated with adaptation of *P. nordicum* to Iberian ham for proposing actions to minimise OTA contamination in this meat product are needed.

Acknowledgements

This work has been funded by the Spanish Ministry of Economy and Competitiveness, Government of Extremadura and FEDER (AGL2013-45729-P, AGL2016-80209-P, and GR15108). Dr. A. Rodríguez is supported by a *Juan de la Cierva*-Incorporación senior research fellowship (IJCI-2014-20666) from the Spanish Ministry of Economy and Competitiveness. Technical support was provided by Facility of Innovation and Analysis in Animal Source Foodstuffs of SAIUEx (SiPA).

References

Andrés, A.I., Ventanas, S., Ventanas, J., Cava, R., Ruiz, J., 2005. Physicochemical changes throughout the ripening of dry cured hams with different salt content and processing conditions. Eur. Food Res. Technol. 221, 30–35. http://dx.doi.org/10.1007/s00217-004-1115-y.

Arnau, J., Guerrero, L., Casademont, G., Gou, P., 1995. Physical and chemical changes in different zones of normal and PSE dry cured ham during processing. Food Chem. 52, 63–69. http://dx.doi.org/10.1016/0308-8146(94)P4182-F.

Asefa, D.T., Gjerde, R.O., Sidhu, M.S., Langsrud, S., Kure, C.F., Nesbakken, T., Skaar, I., 2009. Moulds contaminants on Norwegian dry-cured meat products. Int. J. Food Microbiol. 128, 435–439. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.09.024.

Bernáldez, V., Córdoba, J.J., Andrade, M.J., Alía, A., Rodríguez, A., 2017. Selection of reference genes to quantify relative expression of ochratoxin A-related genes by *Penicillium nordicum* in dry-cured ham. Food Microbiol. 68, 104–111. http://dx.doi.org/10.1016/j.fm.2017.07.001.

Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2009. Predicting mycotoxins in foods: a review. Food Microbiol. 26, 757–769. http://dx.doi.org/10.1016/j.fm.2009.05.014.

Graf, E., Schmidt-Heydt, M., Geisen, R., 2012. HOG MAP kinase regulation of alternariol biosynthesis in *Alternaria alternata* is important for substrate colonization. Int. J. Food Microbiol. 157, 353–359. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.06.004.

Hayes, B.M.E., Anderson, M.A., Traven, A., Van Der Weerden, N.L., Bleackley, M.R., 2014. Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins. Cell. Mol. Life Sci. 71, 2651–2666. http://dx.doi.org/10.1007/s00018-014-1573-8.

Kamala, A., Ortiz, J., Kimanya, M., Haesaert, G., Donoso, S., Tiisekwa, B., De Meulenaer, B., 2015. Multiple mycotoxin co-occurrence in maize grown in three agro-ecological zones of Tanzania. Food Control 54, 208–215. http://dx.doi.org/10.1016/j.foodcont.2015.02.002.

Karolewiez, A., Geisen, R., 2005. Cloning a part of the ochratoxin A biosynthetic gene cluster of *Penicillium nordicum* and characterization of the ochratoxin polyketide synthase gene. Syst. Appl. Microbiol. 28, 588–595. http://dx.doi.org/10.1016/j.syapm.2005.03.008.

Laureati, M., Buratti, S., Giovanelli, G., Corazzin, M., Lo Fiego, D.P., Pagliarini, E., 2014. Characterization and differentiation of Italian Parma, San Daniele and Toscano dry-cured hams: a multi-disciplinary approach. Meat Sci. 96, 288–294. http://dx.doi.org/10.1016/j.meatsci.2013.07.014.

Lin, C.H., Chung, K.R., 2010. Specialized and shared functions of the histidine kinase- and HOG1 MAP kinase-mediated signaling pathways in *Alternaria alternata*, a filamentous fungal pathogen of citrus. Fungal Genet. Biol. 47, 818–827. http://dx.doi.org/10.1016/j.fgb.2010.06.009.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(− Delta Delta C(T)) method. Methods 25, 402–408. http://dx.doi.org/10.1006/meth.2001.1262.

Long, G.L., Winefordner, J.D., 1983. Limit of detection, a closer look at the IUPAC definition. Anal. Chem. 55, 712–724. http://dx.doi.org/10.1021/ac00258a001.

Markov, K., Pleadin, J., Bevardi, M., Vahčić, N., Sokolić-Mihalak, D., Frece, J., 2013. Natural occurrence of aflatoxin B1, ochratoxin A and citrinin in Croatian fermented meat products. Food Control 34, 312–317. http://dx.doi.org/10.1016/j.foodcont.2013.05.002.

Martín, L., Córdoba, J.J., Antequera, T., Timón, M.L., Ventanas, J., 1998. Effects of salt and temperature on proteolysis during ripening of Iberian ham. Meat Sci. 49, 145–153.

Martín, A., Córdoba, J.J., Aranda, E., Córdoba, M.G., Asensio, M.A., 2006. Contribution of a selected fungal population to the volatile compounds on dry-cured ham. Int. J. Food Microbiol. 110, 8–18. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.01.031.

Ministero della Sanità, 1999. Circolare 09.06.1999. In: Sanità, M.D. (Ed.), Circolare 09.06. 1999. vol. 135 Gazzetta Ufficiale, Repubblica Italiana (11.06.1999).

Ostry, V., Malir, F., Toman, J., Grosse, Y., 2016. Mycotoxins as human carcinogens-the IARC monographs classification. Mycotoxin Res. 1–9. http://dx.doi.org/10.1007/s12550-016-0265-7.

Pleadin, J., Staver, M.M., Vahčić, N., Kovačević, D., Milone, S., Saftić, L., Scortichini, G., 2015. Survey of aflatoxin B1 and ochratoxin A occurrence in traditional meat products coming from Croatian households and markets. Food Control 52, 71–77(doi: j.foodcont.2014.12.027).

Rodríguez, A., Rodríguez, M., Luque, M.I., Justesen, A.F., Córdoba, J.J., 2011. Quantification of ochratoxin A-producing molds in food products by SYBR Green and TaqMan real-time PCR methods. Int. J. Food Microbiol. 149, 226–235.

Rodríguez, A., Rodríguez, M., Martín, A., Delgado, J., Córdoba, J.J., 2012. Presence of ochratoxin A on the surface of dry-cured Iberian ham after initial fungal growth in the drying stage. Meat Sci. 92, 728–734. http://dx.doi.org/10.1016/j.meatsci.2012.06.029.

Rodríguez, A., Medina, Á., Córdoba, J.J., Magan, N., 2014. The influence of salt (NaCl) on ochratoxin A biosynthetic genes, growth and ochratoxin A production by three strains of *Penicillium nordicum* on a dry-cured ham-based medium. Int. J. Food Microbiol. 178, 113–119. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.03.007.

Rodríguez, A., Capela, D., Medina, Á., Córdoba, J.J., Magan, N., 2015. Relationship between ecophysiological factors, growth and ochratoxin A contamination of dry-cured sausage based matrices. Int. J. Food Microbiol. 194, 71–77. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.11.014.

Rodríguez, A., Medina, Á., Córdoba, J.J., Magan, N., 2016. Development of a HOG-based real-time PCR method to detect stress response changes in mycotoxigenic moulds. Food Microbiol. 57, 109–115. http://dx.doi.org/10.1016/j.fm.2016.01.008.

Schmidt-Heydt, M., Magan, N., Geisen, R., 2008. Stress induction of mycotoxin biosynthesis genes by abiotic factors. FEMS Microbiol. Lett. 284, 142–149. http://dx.doi.org/10.1111/j.1574-6968.2008.01182.x.

Schmidt-Heydt, M., Graf, E., Batzler, J., Geisen, R., 2011. The application of transcriptomics to understand the ecological reasons of ochratoxin a biosynthesis by *Penicillium nordicum* on sodium chloride rich dry cured foods. Trends Food Sci. Technol. 22, S39–S48. http://dx.doi.org/10.1016/j.tifs.2011.02.010.

Schmidt-Heydt, M., Graf, E., Stoll, D., Geisen, R., 2012. The biosynthesis of ochratoxin A by *Penicillium* as one mechanism for adaptation to NaCl rich foods. Food Microbiol. 29, 233–241. http://dx.doi.org/10.1016/j.fm.2011.08.003.

Schüller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C., Ruis, H., 1994. The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene. EMBO J. 13, 4382–4389.

Singh, K.K., 2000. The *Saccharomyces cerevisiae* SLN1P-SSK1P two-component system mediates response to oxidative stress and in an oxidant-specific fashion. Free Radic. Biol. Med. 29, 1043–1050.

Sonjak, S., Ličen, M., Frisvad, J.C., Gunde-Cimerman, N., 2011. Salting of dry-cured meat - a potential cause of contamination with the ochratoxin A-producing species *Penicillium nordicum*. Food Microbiol. 28, 1111–1116. http://dx.doi.org/10.1016/j.fm.2011.02.007.

Théron, L., Tournayre, P., Kondjoyan, N., Abouelkaram, S., Santé-Lhoutellier, V., Berdagué, J.L., 2010. Analysis of the volatile profile and identification of odour-active compounds in Bayonne ham. Meat Sci. 85, 453–460. http://dx.doi.org/10.1016/j.meatsci.2010.02.015.

Toldrá, F., Aristoy, M.C., 2010. Dry-cured ham. In: Toldrá, F. (Ed.), Handbook of Meat Processing. Wiley-Blackwell, Iowa, pp. 351–362.

Toscani, T., Moseriti, A., Dossena, A., Dall'Asta, C., Simoncini, N., Virgili, R., 2007. Determination of ochratoxin A in dry-cured meat products by a HPLC-FLD quantitative method. J. Chromatogr. B 855, 242–248. http://dx.doi.org/10.1016/j.jchromb.2007.05.010.

Vipotnik, Z., Rodríguez, A., Rodrigues, P., 2017. *Aspergillus westerdijkiae* as a major ochratoxin A risk in dry-cured ham based-media. Int. J. Food Microbiol. 241, 244–251. http://dx.doi.org/10.1016/j.ijfoodmicro.2016.10.031.