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Presence of ochratoxin A on the surface of dry-cured Iberian ham after initial fungal growth in the drying stage

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ABSTRACT

Accumulation of ochratoxin A (OTA) on the surface and to a 0.5 cm depth of dry-cured Iberian ham after initial fungal growth was investigated. For this, 20 dry-cured Iberian hams from the drying stage showing incipient fungal growth on the surface were analyzed. In addition, the presence of OTA-producing molds was examined on the surface of the hams by real-time quantitative PCR (qPCR) based on the *otanps*PN gene. Quantification of specific OTA-producing molds, such as *Penicillium nordicum* and *Penicillium verrucosum* was also achieved on the hams by specific qPCR methods. Ten of 20 dry-cured hams showed OTA at higher levels than those established by legal regulation. OTA was even detected in the deep section of hams. OTA-producing molds ranged from 1.5 to 7.3 log cfu/cm^2. Accumulation of OTA on the hams seems to be related to the presence of OTA-producing molds and especially to *P. nordicum*.

1. Introduction

Dry-cured ham is a meat product ripened for a long time (8–12 months in Serrano type or 24–48 months in Iberian type). The environmental conditions throughout this ripening time, especially in the drying stage, favor growth of a fungal population on the surface of hams, which could include a number of potentially toxigenic molds (Asefa et al., 2009; Dall'Asta et al., 2010; Iacumin et al., 2009; López-Díaz, Santos, García-López, & Otero, 2001; Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996). Mold population growing on the ham surface includes several species, mainly belonging to *Aspergillus* and *Penicillium* genera. Some of them, such as *Aspergillus ochraceus*, *Penicillium nordicum* and *Penicillium verrucosum*, have been reported to be able to produce ochratoxin A (OTA) (Bogs, Battilani, & Geisen, 2006; Iacumin et al., 2009).

OTA is a mycotoxin which has been classified as a possible carcinogen to humans (Group 2B) (IARC, 1993). Several studies have shown it to have teratogenic, neurotoxic, genotoxic, immunotoxic, and nephrotoxic properties (IARC, 1993; Lioi, Santoro, Barbieri, Salsano, & Ursini, 2004). Due to its toxicity, the Commission of the European Communities 2006, in Regulation (EC) 1881/2006 established maximum admissible levels for OTA in several foodstuffs and expressed the appropriateness of setting a maximum level in meat products. However, regulation about the maximum concentration of this mycotoxin on dry-cured hams is only in Italy which limits OTA in this product to 1 μg/kg (Ministero della Sanità, 1999).

OTA production, as with other secondary metabolites, depends on fungal strains as well as growth conditions including substrate composition or physical factors such as pH, water activity or temperature (Giorni, Battilani, Pietri, & Magan, 2008; Molina & Giannuzzi, 2002). In this sense, it is well-known that mycotoxin synthesis is lower in meat substrates than in media with a higher amount of carbohydrates (Bailly, Tabuc, Quérin, & Guerre, 2005; Núñez, Westphal, Bermúdez, & Asensio, 2007; Núñez et al., 2000). In addition, growth of toxigenic molds on the surface of hams does not always indicate the presence of the corresponding mycotoxin (Mateo, Gil-Serna, Patiño, & Jiménez, 2011).

Production of OTA has been reported in several dry-cured meat model systems (Bailly et al., 2005; Battilani, Formenti, Toscani, & Virgili, 2010; Iacumin et al., 2009; Sørensen, Jacobsen, Nielsen, Frisvad, & Koch, 2008). However, there are few data about accumulation of OTA on the surface of hams and its relationship with growth of toxigenic species. Thus, it is necessary to evaluate if initial fungal growth on hams poses a risk of production of OTA. In addition, if OTA is produced by molds growing on the hams throughout the ripening process it could be interesting to evaluate if the accumulation of this mycotoxin is limited to the surface or may penetrate to the inner tissues near to the surface.

OTA production on hams can be determined by using an accurate and sensitive method such as high-performance liquid chromatography (HPLC) (Toscani et al., 2007). Furthermore, from the point of view of food safety programs, it is necessary to quantify potentially OTA-producing molds in the initial fungal population which start to develop on the surface of hams in the drying stage (immediately after the post-salting stage at low temperature). This could be determined using a real time quantitative (qPCR) method that quantifies

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potentially OTA-producing molds (Rodríguez, Rodríguez, Luque, Justesen, & Córdoba, 2011).

All the above information about accumulation of OTA on hams when the fungal growth develops and the presence of potentially OTA-producing molds is of a great interest to evaluate the risk of OTA contamination in hams. In addition, this information could be used to take actions to avoid production of dry-cured hams with OTA.

The purpose of this work was to investigate the accumulation of OTA and the presence of potentially OTA-producing molds on dry-cured Iberian hams, at the beginning of process after initial fungal development.

2. Material and methods

2.1. Sample collection

Twenty dry-cured Iberian hams at the beginning of the drying stage (6 months of ripening) with an initial fungal growth from natural contamination, were analyzed. All hams were derived from Iberian pigs fed with acorn and grass in the country-side (Spanish "dehesa") from a big company with different drying rooms. For sampling of the hams, 25 cm^2 surface areas showing fungal growth and 1 cm of depth, including portions of the *Semimembranosus* and the *Gracilis* muscles (Fig. 1), were excised using a hollow metal sampler with a square cutting edge. The hole in the ham as consequence of sampling was filled with sterilized lard to continue ripening. Mold growth and spores of a superficial square section were scraped off by a sterilized steel scalpel and used for counting by plate and for DNA extraction to quantify potentially OTA-producing molds. Then, the square section was divided into two equal portions (deep and superficial portions) of 0.5 cm of height each (Fig. 1). In both deep and superficial portions, concentration of OTA was determined.

2.2. OTA analysis

2.2.1. OTA extraction

After removing all the mycelia and spores for the microbial analysis and for DNA extraction, the superficial and deep sections were separately macerated for 1 h in a dark flask by shaking with a mixture of 60 mL acetonitrile-water (9:1, v/v) containing 0.1% formic acid and 50 mL hexane. Both superficial and deep sections were weighed before OTA extraction in order to express the amount of mycotoxin detected by weight of sample. The slurry was then transferred to a funnel and the bottom phase was twice filtered through anhydrous sodium sulfate and Whatman no. 1 filter paper (Whatman, Tokyo, Japan). Then, the filtrate was mixed with 50 mL of hexane, shaken for 30 min in the dark and transferred to a Justesen funnel. The bottom phase was twice filtered and the filtrate was evaporated in a rotatory evaporator model VV2000 (Heidolph, Kelheim, Germany) at 40–45 °C. The residue was resuspended in 1 mL of chloroform, filtered through a 0.45 μm nylon membrane (MSI, Westboro, Mass.), and evaporated to dryness under a gentle stream of nitrogen. The extracts were briefly stored at 4 °C in the dark before high-performance liquid chromatography–mass spectrometry (HPLC–MS) analysis.

This method was initially validated by analysis of Iberian dry-cured ham spiked with different amounts of OTA (2.0, 10.0 and 20.0 ng/g). The OTA recovery with the above method was always higher than 90%.

2.2.2. OTA detection by HPLC–MS

Mycotoxin extracts were resuspended in 100 μL of HPLC-grade acetonitrile (Sharlab, Barcelona, Spain) before HPLC–MS analysis. OTA production in the depth and on the surface of dry-cured samples was analyzed by HPLC–MS following as described by Sosa et al. (2002) with some modifications. Analyses were carried out in a Single

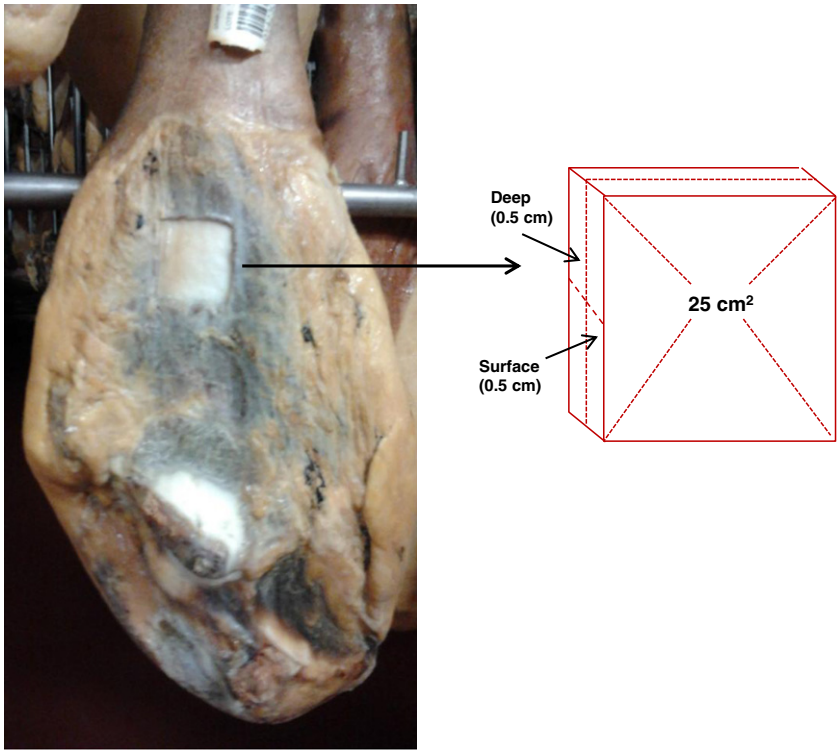

Fig. 1. Sampling of dry-cured Iberian ham from the drying stage showing initial fungal growth. A schematic representation of the 25 cm^2 surface areas showing 1 cm of depth taken in the sampling is also shown. After sampling the hole in the ham was filled with sterilized lard to continue the ripening.

Quadrupole Agilent G1946B HPLC–MS system (Agilent Technologies, Santa Clara, CA, USA), using a C18 column (25 cm long, 4.6 mm inside diameter and 5 μm particle size, SUPELCO, Bellafonte, USA) with mobile phases (A) 100% water and (B) 0.05% trifluoroacetic acid in acetonitrile in a gradient from 10% to 99%. Detection of OTA was carried out in a mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source in the SIM (Single Ion Mode) mode according to retention time and molecular mass. Under these conditions, OTA eluted at 16.5 min as a protonated molecular ion of m/z 404. The calibration curve for OTA by HPLC–MS revealed a linear relationship ($R^2 = 0.9709$) between detector response and amount of OTA standard (Sigma-Aldrich Química S.A., Madrid, Spain) from 1 to 100 ng. The full MS spectra data were acquired and processed using the Agilent Technologies software. The minimum detectable value or limit of detection (LOD) was estimated from the calibration curve, according to the equation: $LOD = 3\ (s_B^2 + s_i^2 + (i/m)^2\ s_m)^{1/2}/m$ (Long & Winefordner, 1983) where m is the slope of the calibration curve, i is the intercept term, s_B, s_i and s_m are the standard errors of the blank, the intercept term and the slope of the calibration curve, respectively.

Assuming a normal distribution of the estimated quantities, α (error of the first type) = β (error of the second type) = 0.05, the quantification limit (LOQ) was 3.04 LOD (IUPAC, 1995). The HPLC–MS method used for the OTA analysis on dry-cured Iberian ham showed a LOD of 1 μg/kg and a LOQ of 3 μg/kg.

2.3. Fungal count estimation

All the mycelium taken from the square section of the dry-cured Iberian ham sample was homogenized with 9 mL of 0.1% sterile peptone in water. From this suspension, 1 mL was taken for count estimation by plating in culture medium and the remaining 9 mL was used for DNA extraction to be analyzed in qPCR assays. With the 1 mL fraction, 10-fold dilutions were also prepared in 0.1% sterile peptone water. A volume of 100 μL of these solutions was plated out on Potato Dextrose Agar (PDA, Sharlau Chemie S.A., Spain). These plates were incubated at 25 °C for 4 days. Then, colonies were counted and the number of cfu/cm^2 calculated.

2.4. qPCR assays

2.4.1. DNA extraction

The remaining suspension was processed according to the "CTAB-EZNA" method previously optimized by Rodríguez, Rodríguez, et al. (2012).

2.4.2. Duplex TaqMan qPCR for quantifying total OTA-producing molds

To quantify potentially OTA-producing molds in the DNA samples from dry-cured hams, the F/R-npstr primer pair and *NPSprobe* probe designed on a conserved region of the *otanps*PN gene and non-competitive IAC primers and probe set (TubF1/TubR1 and *Tubprobe*) based on the housekeeping highly conserved *β-tubulin* gene reported by Rodríguez et al., 2011; Rodríguez, Córdoba, et al. (2012) were used (Table 1). The duplex qPCR was carried out in the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). The qPCR was prepared in triplicate in a final volume of 25 μL reaction mixture in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). Three replicates of a control sample without DNA template were also included in all runs. A mixture containing 5 μL of genomic DNA from the OTA producing strain *P. nordicum* CBS 110769 was used to optimize the duplex qPCR method. For optimizing the concentration of primers and probes, several concentrations ranging from 50 to 600 nM were assayed. The optimized TaqMan protocol was carried out in a final volume of 25 μL, containing 5 μL of template DNA, 12.5 μL of 2× *Premix Ex Taq*™ (Takara, Otsu, Shiga, Japan), 0.5 μL of 50× ROX™ Reference Dye (Takara), 80 nM of both TubF1 and TubR1 primers and *Tubprobe*, and 400 nM of both F-npstr and R-npstr primers and *NPSprobe*.

The thermal cycling conditions included an incubation of 2 min at 50 °C to allow the activation of uracil-*N*-glycosylase (UNG) enzyme, an incubation step for 10 min at 95 °C to denature the UNG enzyme and activate AmpliTaq Gold polymerase, 40 cycles at 95 °C for 15 s, 59 °C for 30 s and 61 °C for 30 s. Threshold cycle (Ct) determinations were automatically performed by the instrument using default parameters.

To calculate the fungal load, two standard curves (each one for total fungal and for OTA-producing molds) were constructed with the DNA extracted from sterile dry-cured ham inoculated with the OTA producing strain *P. nordicum* CBS 110769 as previously described (Rodríguez et al., 2011). For this, the spore suspension obtained from the above producing strain was filtered through Whatman paper No 1 and quantified by microscopy, using a Thoma counting chamber. Aliquots of 1 mL of 10^2, 10^3, 10^4, 10^5, and 10^6 conidia/mL of this mold strain were immediately used to inoculate dry-cured ham, to concentrations of 1, 10, 10^2, 10^3, and, 10^4 cfu/cm^2 of food. Next, the DNA extraction from inoculated hams was performed as indicated in Section 2.4.1. All inoculations, extractions and qPCR analyses were carried out in triplicate. In addition, triplicates of non-inoculated negative control of hams were included in each experiment. Thus, the standard curves relating the Ct values and the log cfu/cm^2 values of the inoculated hams using the *otanps*PN and IAC primers were generated.

2.4.3. qPCR assays to quantify specific OTA-producing mold species

The DNA extracted from the dry-cured hams was also used for the quantification of typical *Penicillium* and *Aspergillus* OTA-producing species by specific qPCR methods. For this, specific primers based on the *ochratoxin A polyketide synthase* gene (*otapks*PN) previously described, were used to detect three different OTA-producing species (Table 1): *P. nordicum* (Geisen, Mayer, Karolewiez, & Färber, 2004), *P. verrucosum* (Schmidt-Heydt, Richter, Michulec, Buttinger, & Geisen, 2008) and *A. niger* aggregate (also *A. ochraceus*) (Castellá & Cabañes, 2011). The

Table 1
Nucleotide sequence of primers and probe used for either SYBR Green or TaqMan Real Time PCR assays.

Primer name	Nucleotide sequences (5′–3′)	Target gene	Target specie	Reference
F-npstr	GCCGCCCTCTGTCATTCCAAG	*otanps*PN	Total OTA-producing molds	Rodríguez et al. (2011)
R-npstr	GCCATCTCCAAACTCAAGCGTG			
NPSprobe	[Cy5]-CGGCCGACCTCGGGAGAGA-[BHQ2]			
TubF1	TCCCTTCGGCAAGCTTTTC	*β-Tubulin*	Total molds	Rodríguez, Córdoba, et al. (2012)
TubR1	TGTTACCAGCACCGGACTGA			
Tubprobe	[CY3]-CGCCCCGACAACT-[BHQ2]			
Otapkstaq1	CACGGTTTGGAACACCACAAT	*otapksPN*	*P. nordicum*	Geisen et al. (2004)
Otapks2	TGAAGATCTCCCCCGCCT			
Otapkspv1sybr1-for	TTGCGAATCAGGGTCCAAGTA	*otapksPN*	*P. verrucosum*	Schmidt-Heydt et al. (2008)
Otapkspv1sybr1-rev	CGAGCATCGAAAGCAAAAACA			
ANPKSFRT	ATCTCTCGGCAGGCAGCAT	*otapksPN*	*A. niger (A. ochraceus)*	Castellá and Cabañes (2011)
ANPKSRRT	CACTCGCTCCAGTTGATGGA			

specific qPCR assays were performed in the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). The SYBR Green methodology was used. Amplification reactions were carried out in triplicate in a final volume of 25 μL containing 5 μL of template DNA, 12.5 μL of 2× SYBR® *Premix Ex Taq*™ (Takara), 0.5 μL of 50× ROX™ Reference Dye and 1.5 μL of each primer (10 μM).

The amplification program used was: 1 cycle of 10 min at 95 °C and 40 cycles of 95 °C for 15 s and 60 °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. Ct determinations were automatically performed by the instrument using default parameters.

To calculate the specific fungal load, three standard curves were constructed with the DNA extracted from sterile dry-cured ham inoculated with each one of typical OTA-producing species (*P. nordicum* CBS 110769, *P. verrucosum* CBS 323.92 and *A. ochraceus* CBS 589.68). This analysis was carried out in the same way as described in Section 2.4.2. The standard curve for quantifying potentially OTA-producing molds was obtained from inoculated dry-cured ham slices with *P. nordicum* CBS 110769. Thus, three standard curves relating the Ct values and log cfu/cm^2 of the dry-cured ham samples using the otapkstaq1–otapks2, otapksPVSYBR1-for–otapksPVSYBR1-rev and ANPKSFRT–ANPKSRRT primer pairs for quantifying *P. nordicum*, *P. verrucosum* and *A. ochraceus* in the ham samples were generated. All extractions and qPCR analyses were performed in triplicate. In addition, triplicates of non-inoculated negative control of hams were included in each experiment.

2.5. Statistical analysis

Normality of distribution of the data was tested by the Kolmogorov–Smirnov test and all of them showed a normal distribution. All the statistical analyses were performed with the SPSS v.15.0. One way analysis of variance (ANOVA) was carried out to determinate significant differences within and between groups. Tukey's test was applied to compare the mean values. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. OTA in the dry-cured Iberian ham samples

The results obtained from the determination of OTA in the 20 dry-cured Iberian ham samples (superficial and deep sections) are included in Table 2. 10 of 20 dry-cured Iberian hams were contaminated with OTA above the LOD (1 μg/kg) and 3 of the 10 positive samples (5, 15 and 19) showed OTA in the deep section. Samples 15 and 19 showed the highest OTA concentrations on the surface and in the deep sections, respectively. The quantity of this mycotoxin detected on the most superficial section of the hams ranged from 2.0 to 160.9 μg/kg. In addition, five hams had surface concentration of OTA higher than 15 μg/kg.

3.2. Quantification of fungal load on dry-cured ham by counting plate and qPCR

The fungal growth on the 20 dry-cured Iberian hams was first determined by counting plate (Table 3). Similar total numbers of molds was recorded in all ham samples. These counts ranged between 6.8 and 7.5 log cfu/cm^2.

The fungal load (cfu/cm^2) obtained by qPCR using primers (TubF1/TubR1) based on the β-*tubulin* gene is shown in Table 3. The counts ranged from 5.4 to 7.3 log cfu/cm^2. In general, there was nearly a 1:1 correlation between the cfu data determined by plate counting and cfu data determined by the qPCR assay (Table 3).

Table 2
Levels of ochratoxin A detected in samples of dry-cured Iberian ham at the ripening stage (after 6 months of ripening) showing initial fungal growth.

Sample reference of Iberian ham	Average ochratoxin A concentration (μg/kg)	
	Superficial portion	Deep portion
1	nd	nd
2	nd	nd
3	nd	nd
4	2.2 ± 0.07	nd
5	2.1 ± 0.18	2.0 ± 0.10
6	nd	nd
7	nd	nd
8	17.3 ± 0.31	nd
9	15.1 ± 0.16	nd
10	6.5 ± 0.22	nd
11	nd	nd
12	nd	nd
13	nd	nd
14	13.0 ± 0.42	nd
15	160.9 ± 0.48	18.4 ± 0.13
16	nd	nd
17	2.0 ± 0.12	nd
18	22.7 ± 0.38	nd
19	29.2 ± 0.43	28.4 ± 0.12
20	nd	nd

nd: ochratoxin A was not detected.

3.3. Quantification of potentially OTA-producing mold species in dry-cured ham by qPCR

The qPCR involving the primer pairs, F-npstr and R-npstr, was used to detect and quantify potentially OTA-producing molds in the dry-cured ham samples. The standard curve was $y = -3.39\ x + 42.09$, $R^2 = 0.99$ with $P \leq 0.05$. The detection limit of the qPCR was 1 log cfu/cm^2. All the tested dry-cured hams had mean values of OTA-producing molds (Table 3) ranging from 1.5 to 7.3 log cfu/cm^2. Several dry-cured hams, such as 1, 2, 14, 15 and 19 showed similar counts of total fungal and OTA-producing molds. All samples contaminated with OTA showed counts of potentially OTA-producing molds above 4.4 log cfu/cm^2.

3.4. Quantification of specific potentially OTA-producing mold species in dry-cured ham by qPCR

When qPCR with the specific primer pair to quantify *P. nordicum* was tested, one PCR product of the expected size of 68 bp showing a Tm of 82.6 ± 0.3 °C was found only when DNA from this specie was present. The standard curve was $y = -3.30\ x + 42.24$, $R^2 = 0.99$ with $P \leq 0.05$ and the detection limit of the qPCR was 1 log cfu/cm^2. Counts of *P. nordicum* in the samples ranged from 2.5 to 5.3 log cfu/cm^2. Sample 15 showed the highest count of *P. nordicum* (5.3 log cfu/cm^2). 10 of 19 samples contaminated with *P. nordicum* showed contamination with OTA the minimum counts of *P. nordicum* being 3.2 log cfu/cm^2.

The detection of the OTA-producing *P. verrucosum* yielded one PCR product of the expected size of 51 bp showing a Tm of 79.2 ± 0.3 °C, only when DNA from *P. verrucosum* was present. The standard curve was $y = -3.99\ x + 43.12$, $R^2 = 0.98$ with $P \leq 0.05$ and the detection limit of the qPCR was 1 log cfu/cm^2. *P. verrucosum* was detected in 17 samples. Counts of *P. verrucosum* were significantly lower ($P \leq 0.05$) than those obtained for *P. nordicum*, and ranged from 1.3 to 2.2 log cfu/cm^2. Samples 14, 17 and 18 showed the highest counts of *P. verrucosum*. 10 of 17 samples contaminated with *P. verrucosum* showed contamination with OTA. However, no significant correlation ($P > 0.05$) was found between OTA level on the surface and the number of cfu/cm^2 of *P. verrucosum* or between counts of specific OTA producing species and total OTA producers determined by qPCR.

Table 3
Quantification of total fungal growth by plate (log cfu/cm^2) and by qPCR and quantification of potentially ochratoxigenic fungal growth by qPCR.

Sample reference	Average total count (log cfu/cm^2)			Average ochratoxigenic counts by specific qPCR assays (log cfu/cm^2)					
	Fungal load by plate	Fungal load by qPCR	Recovery rate (%)	OTA-producers	*P. nordicum*	*P. verrucosum*	*A. ochraceous*	*P. nordicum*/ *OTA-producers*	*P. verrucosum*/ *OTA-producers*
1	6.8 ± 0.42	6.8 ± 0.12	100	6.1 ± 0.02	4.3 ± 0.08	2.0 ± 0.12	–	0.71	0.33
2	7.1 ± 0.10	6.6 ± 0.16	92.7	6.1 ± 0.04	4.4 ± 0.03	2.1 ± 0.04	–	0.72	0.34
3	7.1 ± 0.28	5.8 ± 0.18	81.0	3.9 ± 0.09	2.5 ± 0.01	<1.0	–	0.64	–
4	6.8 ± 0.38	6.1 ± 0.09	89.9	5.3 ± 0.06	3.9 ± 0.12	1.9 ± 0.08	–	0.73	0.36
5	6.8 ± 0.15	6.4 ± 0.15	93.9	5.8 ± 0.11	3.9 ± 0.46	1.8 ± 0.05	–	0.67	0.31
6	7.2 ± 0.76	5.7 ± 0.13	79.2	4.3 ± 0.16	3.2 ± 0.13	1.4 ± 0.09	–	0.74	0.32
7	7.1 ± 0.60	6.3 ± 0.24	74.8	5.7 ± 0.88	4.4 ± 0.29	1.9 ± 0.17	–	0.77	0.33
8	6.9 ± 0.53	5.4 ± 0.21	88.3	4.4 ± 0.04	3.2 ± 0.13	1.4 ± 0.19	–	0.72	0.32
9	7.2 ± 0.39	6.9 ± 0.06	78.6	6.2 ± 0.05	4.3 ± 0.01	2.0 ± 0.08	–	0.69	0.32
10	7.5 ± 0.41	6.5 ± 0.05	95.6	5.5 ± 0.15	3.9 ± 0.31	1.9 ± 0.70	–	0.71	0.35
11	7.5 ± 0.35	6.1 ± 0.10	87.4	5.5 ± 0.03	3.3 ± 0.08	1.3 ± 0.15	–	0.60	0.24
12	7.1 ± 0.29	5.8 ± 0.15	79.7	4.7 ± 0.10	3.7 ± 0.43	1.7 ± 0.05	–	0.79	0.36
13	7.4 ± 0.41	7.3 ± 0.23	82.2	5.7 ± 0.52	4.6 ± 0.01	2.1 ± 0.11	–	0.80	0.37
14	7.2 ± 0.37	7.0 ± 0.04	98.3	6.9 ± 0.07	3.8 ± 0.14	2.2 ± 0.01	–	0.55	0.32
15	7.0 ± 0.61	6.5 ± 0.07	98.7	6.6 ± 0.06	5.3 ± 0.09	1.7 ± 0.01	–	0.80	0.26
16	7.7 ± 0.42	6.9 ± 0.04	91.9	5.6 ± 0.11	3.0 ± 0.11	<1.0	–	0.53	–
17	7.4 ± 0.18	7.5 ± 0.09	87.7	5.8 ± 0.10	4.3 ± 0.25	2.2 ± 0.12	–	0.74	0.38
18	6.8 ± 0.21	6.7 ± 0.04	100	6.4 ± 0.10	4.7 ± 0.05	2.2 ± 0.02	–	0.73	0.34
19	7.4 ± 0.24	7.2 ± 0.09	97.6	7.3 ± 0.23	4.6 ± 0.10	1.5 ± 0.05	–	0.63	0.21
20	7.4 ± 0.28	7.2 ± 0.16	98.1	1.5 ± 0.30	<1.0	<1.0	–	–	–

All data represent the mean amount of fungal load quantified (log cfu/cm^2) ± standard deviation (SD) of the 3 independent experiments each consisting of triplicate samples.

When qPCR for testing the presence of *A. niger* aggregate and *A. ochraceous* was assayed, a PCR product of the expected size of 120 bp showing a Tm of 80.1 ± 0.2 °C was detected when genomic DNA of *A. ochraceus* CBS 589.68 was used. However, no qPCR amplification was observed in any of the 20 dry-cured hams.

4. Discussion

In the present work, the relationship between the presence of different potentially OTA-producing species and OTA production in dry-cured Iberian hams with an initial fungal growth at the beginning of process (6 months of ripening) has been evaluated.

A HPLC–MS method was used to determine OTA on the hams. This method has been previously used for an accurate detection and quantification of mycotoxins in several foods (Al-Hazmi, 2010; Brera, Grossi, & Miraglia, 2005; Gonçalez et al., 2008). The LOD and the LOQ values obtained were lower than those reported for the analysis of this mycotoxin by HPLC–MS (Sørensen et al., 2010). These values provide information about the reproducibility and the minimum detectable value of the method (Toscani et al., 2007).

From the results, up to 50% of the dry-cured hams contained OTA above the LOD. Concentrations of OTA found on the surface for most of hams, was higher than the 3.62 μg/kg previously reported for fully ripened dry-cured ham by Dall'Asta et al. (2010). Although no international guidelines have been established for the maximum OTA level allowed for these kinds of products, the OTA amount in these hams was higher than the legal limit of OTA for pork meat and derived products (1 μg/kg) in the Italian legislation (Ministero della Sanità, 1999). In addition it is noticeable that 30% of the hams that showed OTA on the surface also contained this mycotoxin in the inner tissues at levels above 1.9 μg/kg. That means that OTA penetrates to inner tissues of the hams. Up to now, OTA has been reported in the deep muscle tissues obtained from pigs fed with OTA contaminated feeds (Dall'Asta et al., 2010). However, in the present work, OTA cannot derive from feed, since all the dry-cured hams came from Iberian pigs fed with acorn and grass in the country-side (Spanish "dehesa"). In addition, there are differences in the OTA concentrations between the surface and the inner tissue, showing in most cases the lowest values in the depth. These results are of special relevance, since they are observed in dry-cured hams at the beginning of processing and after the development of fungal growth on the surface. Iberian dry-cured hams are usually aged for 24 months at 15–25 °C (Núñez et al., 1996). During this time, dry-cured ham is frequently heavily covered by fungal mycelium. Thus, it can be assumed that OTA accumulation will be much higher in fully ripened dry-cured hams. Therefore, it would be necessary to take preventive actions such as use of protective non OTA-producing molds and the control of temperature or relative humidity. In addition, the use of a rapid method such as qPCR may be of great value in the monitoring or verification procedures to quantify toxigenic molds in HACCP plans. Thus, when OTA-producing molds are detected in hams during processing (drying stage), corrective actions such as the elimination of mycelium from the surface of the hams by washing and the subsequent application of a protective culture of non toxigenic molds, could be taken to avoid the processing of fully ripened dry-cured hams contaminated with OTA.

The quantification of total fungal growth in dry-cured hams by qPCR is in concordance with that obtained by plate, since the recovery rates were in most of cases approaching 100% indicating that the qPCR method is a good tool for estimating the fungal load of hams. The presence of potentially ochratoxigenic molds on dry-cured ham was tested by using the qPCR based on the *otanps*PN gene (Rodríguez et al., 2011), and modified by the inclusion of an IAC. In this way, a control signal is amplified together with that obtained of the target sequence under the same conditions. This shows that there is no failure due to the thermal cycler, incorrect PCR mixture, poor DNA polymerase activity, or the presence of inhibitory substances in the sample matrix (Radström, Löfström, Lövenklev, Knutsson, & Wolffs, 2003).

In general, high counts of potentially ochratoxigenic molds were observed, being close in some cases to the total counts of molds present in these samples. This explains the high levels of OTA accumulation found. However, the highest count of potentially OTA-producing molds did not mean the highest OTA accumulation on the surface. However, this implied the highest OTA accumulation in the depth. Probably the accumulation of OTA on hams is not only related to the number of OTA-producing molds, but also to the OTA-producing species present in these samples. Thus, specific fungal identification is essential to provide information about which mycotoxins could be produced in foods (Lindblad, Johnsson, Jonsson, Lindqvist, & Olsen, 2004; Maenetje & Dutton, 2007; Magnoli, Astoreca, Chiacchiera, & Dalcero, 2007; Medina, Mateo, López-Ocaña, Valle-Algarra, & Jiménez, 2005; Medina et al., 2006). In this work, OTA-producing *Penicillium* spp.

(*P. nordicum* and *P. verrucosum*) were found at detectable levels in all dry-cured samples where OTA was detected. However, counts of *A. niger* aggregate and *A. ochraceous* were in all cases below the detection limit of the qPCR methods. In agreement with these results, *P. nordicum* has been reported frequently on dry-cured meat products and other ripened foods such as salted cheeses (Lund & Frisvad, 2003; Schmidt-Heydt, Graf, Batzler, et al., 2011; Sonjak, Ličen, Frisvad, & Gunde-Cimerman, 2011). Also, these species have been isolated from several dry-cured ham and sausage processing plants (Battilani et al., 2010; Bogs et al., 2006; Iacumin et al., 2009). With regard to *P. verrucosum*, it is mainly adapted to cereals, but can occasionally be found on dry-cured ham (Andersen, 1995; Comi, Orlic, Redzepovic, Urso, & Iacumin, 2004; Peintner, Geiger, & Pöder, 2000). The difference of counts found in both *Penicillium* species ($P \leq 0.05$) could be explained by the higher capacity of adaptation of *P. nordicum* to sodium chloride rich foods (Schmidt-Heydt, Graf, Stoll, et al., 2011). Other OTA producing *Aspergillus* species such as *A. carbonarius*, *A. niger*, *A. ochraceus*, *A. westerdjkiae* and *A. steynii* (Belli et al., 2005; Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2011; Leong et al., 2007; Varga & Kozakiewicz, 2006) are specially adapted to various food commodities like grapes, coffee, cocoa or spices (Bayman & Baker, 2006). However, to date none of these species has been described as a contaminant in dry-cured meat products (Schmidt-Heydt, Graf, Batzler, et al., 2011).

The highest counts of *P. nordicum* related to a major OTA concentration on the surface. This makes sense as most strains of *P. nordicum* are strong and constant OTA producers (Larsen, Svendsen, & Smedsgaard, 2001; Lund & Frisvad, 2003). However, *P. verrucosum* produces generally less and more inconsistent OTA (Schmidt-Heydt, Graf, Stoll, et al., 2011). In previous studies, Iacumin et al. (2009) demonstrated that some dry-fermented sausages contaminated with OTA were due to the presence of both *P. verrucosum* and *P. nordicum* species. However, others were due to the presence of *P. nordicum* as the only OTA producer. These findings are consistent with those reported by Larsen et al. (2001) and Pitt (1987), both of whom demonstrated that *P. nordicum* and *P. verrucosum* are the two main *Penicillium* species responsible for OTA production.

Counts of potentially OTA producing molds and the ochratoxigenic *P. nordicum* (both quickly determined by qPCR) may be used as indicators to predict the risk of OTA accumulation on dry-cured Iberian ham. However, *P. verrucosum* did not show any correlation with OTA accumulation in the samples of dry-cured Iberian ham ($P > 0.05$).

The qPCR-based strategy in this work is the first time that it has been used to study the occurrence of ochratoxigenic molds in dry-cured hams. Up to now, conventional PCR based strategies have been used to detect contamination with mycotoxigenic *Fusarium* species in naturally contaminated maize (Jurado, Vázquez, Marín, Sanchís, & González-Jaén, 2006), and to detect contamination with aflatoxigenic and ochratoxigenic *Aspergillus* spp. in barley grain (Mateo et al., 2011).

In conclusion, OTA was detected on dry-cured Iberian hams at the beginning of processing and after initial fungal growth. In addition, the presence of OTA is not limited to the surface and penetrates to the inner tissues. The application of the qPCR assays for detection and quantification of ochratoxigenic molds and *P. nordicum* could be considered a good tool for routine analysis in HACCP programs to prevent and control the risk of accumulation of OTA in the production of Iberian dry-cured ham.

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