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Evaluation of hazard of aflatoxin B1, ochratoxin A and patulin production in dry-cured ham and early detection of producing moulds by qPCR

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ABSTRACT

The growth of aflatoxin B1 (AFB1), ochratoxin A (OTA) and patulin producing strains and the hazard of their mycotoxins production in dry-cured ham were evaluated. In addition, effectiveness of real-time quantitative PCR (qPCR) for the detection and quantification of toxigenic moulds in this product before mycotoxins production was analyzed. For this, slices of dry-cured ham were inoculated in separate assays with AFB1, OTA and patulin producers and incubated at 97% and 84% relative humidity (RH) for 21 days at 25 °C. Higher counts were reached by AFB1 and patulin producing species at 97% RH, and by OTA producing species at 84% RH. The production of AFB1, OTA and patulin on dry-cured ham slices was demonstrated. The estimation of toxigenic moulds by qPCR was highly correlated with the counts obtained by plating in culture media. All the qPCR protocols assayed detected and quantified toxigenic moulds in the hams before mycotoxins were produced. Thus, qPCR should be considered for a reliable and rapid quantification of toxigenic moulds in dry-cured ham.

1. Introduction

Mycotoxins are secondary metabolites from filamentous fungi, which can be produced in foods as a result of fungal growth when environmental conditions are appropriated. The toxicity and carcinogenicity of some mycotoxins, and their potential to contaminate foods is a cause of serious concern globally, both from a food safety and food trade standpoint (Edwards, O'Callaghan, & Dobson, 2002). Among these compounds, aflatoxin B1 (AFB1), ochratoxin A (OTA) and patulin are mycotoxins extremely toxic with carcinogenic and teratogenic effects (Beretta, Gaiaschi, Galli, & Restani, 2000; Mayer, Bagnara, Färber, & Geisen, 2003; Schmidt-Heydt, Abdel-Hadi, Magan, & Geisen, 2009). They are synthesized by a variety of mould. Thus, aflatoxins are produced by moulds from the genus *Aspergillus* (Mayer et al., 2003; Schmidt-Heydt et al., 2009), patulin by moulds belonging to the genera *Byssochlamys* and *Penicillium* (Niessen, 2007; Sant'Ana et al., 2010), and OTA by species of the genera *Aspergillus* and *Penicillium* (Bogs, Battilani, & Geisen, 2006; Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2011).

These moulds may grow in some foods when the appropriate ecological conditions are found. In dry-ripened foods such as dry-cured ham or dry-fermented sausages, the environmental conditions throughout the ripening process favour the growth of a mould population composed mainly by *Penicillium* and *Aspergillus* spp. including a high rate of potentially toxigenic moulds (Asefa et al., 2009; Iacumin et al., 2009; López-Díaz, Santos, García-López & Otero, 2001; Núñez, Rodríguez, Bermúdez, Córdoba, & Asensio, 1996). Several strains isolated from dry-cured meat products have been reported as AFB1, OTA and patulin producers (Bogs et al., 2006; Iacumin et al., 2009; Martín, Jurado, Rodríguez, Núñez, & Córdoba, 2004). However, the environmental conditions for mycotoxin production are generally more restricted than those for mould growth (Frisvad & Samson, 1991). In addition, the toxigenic potential of moulds is directly dependent on the substrate on which they are grown, and mycotoxin synthesis is lower in meat substrates than in media with a higher amount of carbohydrates (Bailly, Tabuc, Quérin, & Guerre, 2005; Núñez et al., 2000; Núñez, Westphal, Bermúdez, & Asensio, 2007). This highlights that the growth of the toxigenic moulds on the surface of hams does not always indicate the presence of corresponding mycotoxin (Mateo, Gil-Serna, Patiño, & Jiménez, 2011). However, OTA and AFB1 have been reported in dry-cured meat models as a consequence of the growth of the producing strains (Battilani, Formenti, Toscani, & Virgili, 2010; Iacumin et al., 2009; Rojas, Jodral, Gosalvez, & Pozo,

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1991). In addition, OTA was reported in dry-cured hams from pigs fed with OTA contaminated diet (Dall'Asta et al., 2010). Although the presence of patulin has not been described in dry-cured ham, some producing strains have been isolated in this product throughout the ripening process (Martín et al., 2004). Therefore, it should be elucidated if the presence of AFB1, OTA and patulin producing strains on dry-cured hams suppose a risk of mycotoxins contamination of this product.

Mould growth on the hams occurs mainly at the drying and cellar stages and even during storage of final product, where temperature reaches until 25 °C. At this temperature, relative humidity (RH) may affect growth of the moulds and the production of toxins. Thus, it is necessary to evaluate the effect of RH on mycotoxins accumulation on the hams. In addition, the analysis of dynamic of mycotoxins production may be of great interest to know if it is possible an early detection of toxigenic moulds before toxins were produced.

Detection of toxigenic moulds by DNA-based techniques, such as real-time quantitative PCR (qPCR), is a good alternative to traditional detection techniques. This technique is a specific and highly sensitive, since the mould DNA target can be detected in complex mixtures (González-Salgado, Patiño, Gil-Serna, Vázquez, & González-Jaén, 2009; Mateo et al., 2011). Several qPCR assays have been developed to detect and quantify AFB1, OTA and patulin producing moulds in foods using as target constitutive genes, toxin biosynthesis genes or multi-copy sequences (Gil-Serna, Gónzalez-Salgado, Gónzalez-Jaén, Vázquez, & Patiño, 2009; Mayer et al., 2003; Passone, Rosso, Ciancio, & Etcheverry, 2010; Sardiñas, Vázquez, Gil-Serna, Gónzalez-Jaén, & Patiño, 2011; Schmidt-Heydt et al., 2009). Thus, the sterigmatocystin O-methyltransferase (*omt-1*), the non-ribosomal peptide synthetase (*otanps*PN) and the isoepoxydon dehydrogenase (*idh*) genes involved in aflatoxin, OTA and patulin biosynthesis respectively, have been successfully used in qPCR assays (Rodríguez, Luque et al., 2011; Rodríguez, Rodríguez, Gordillo, Andrade, & Córdoba, 2011; Rodríguez, Rodríguez, Luque, Justensen, & Córdoba, 2011). In order to be used in HACCP programs for dry-cured hams, the utility of these qPCR methods to quantify toxigenic moulds before mycotoxin production should be tested.

The purpose of this work was to study the growth of AFB1, OTA and patulin producing strains in dry-cured ham to elucidate if their presence supposes a hazard of mycotoxins contamination. In addition, the dynamic of production of these mycotoxins on ham at different environmental conditions has been analyzed to evaluate the possibility of early detection, by using qPCR of moulds before mycotoxins were produced.

2. Material and methods

2.1. Fungal strains

Six mould strains, belonging to the Spanish Type Culture Collection (CECT), the Centraalbureau voor Schimmelcultures in The Netherlands (CBS) and the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark (IBT) were used: *Aspergillus flavus* IBT 3696 and *Aspergillus parasiticus* CECT 2688 as AFB1-producing strains, *Penicillium nordicum* CBS 110769 and *Aspergillus ochraceus* CBS 569.68 as OTA producing strains and *Penicillium expansum* CECT 2278 and *Penicillium griseofulvum* CBS 110.420 as patulin producing strains. All of them were established as AFB1, OTA and patulin producers by their respective Culture Collections.

2.2. Sample preparation

Slices of commercial non sterile dry-cured ham (30.5 g proteins, 4.5 g fat, 3.81 g NaCl per 100 g of product; pH 6) with a cut surface of 25 cm^2 and approximately 5 g of weight were aseptically prepared and placed separately in pre-sterilized orthogonal receptacles made of methacrylate, where the humidity was kept constant after vapour–liquid equilibrium with saturated salts solutions placed at the bottom of the receptacles. Two different RH were thus achieved in the receptacles where dry-cured ham slices were incubated:

a) 97% RH generated by a saturated K_2SO_4 solution. b) 84% RH generated by saturated KCl solution.

Water activity (A_w) of ham slices places in the above RH was measured with a Novasina Lab Master from Novasina AG (Lachen, Switzerland) at 0, 7, 14 and 21 days of incubation. Ham slices incubated at 97% RH showed constant values of A_w throughout the incubation time at 0.92 ($\pm$0.003). Similar A_w values have been reported in external muscles (*Gracilis* or *Semimebranosus*) of dry-cured ham at the end of post-salting or in drying stages (Andrés, Ventanas, Ventanas, Cava, & Ruiz, 2005; Rodríguez et al., 1994). Ham slices incubated at 84% RH showed A_w values of 0.84 ($\pm$0.014) throughout the incubation time. These values of A_w have been reported in external muscles (*Gracilis* or *Semimebranosus*) of dry-cured ham at the end of drying or in cellar stages (Andrés et al., 2005; Rodríguez et al., 1994). Moulds grow on hams mainly in drying and cellar stages when the surface reaches the former A_w values. This fact justifies the two environmental RH selected for the incubation tests, which allow keep the ham slices at similar A_w values reached during drying (0.92) or cellar (0.84) stages.

Dry-cured ham slices placed in the above RH were inoculated separately on the surface by spreading conidia suspensions of each selected moulds at a concentration which has been used previously by Mayer et al. (2003) for contamination studies of 3 log spores per gram of dry-cured ham. Sampling was carried out by triplicate from each at 0, 7, 14 and 21 days of incubation at 25 °C. Negative controls from non-inoculated slices of dry-cured ham were also analyzed in each RH and sampling tested.

Samples (5 g) taken in the different sampling times were homogenized with 10 mL of Tris–HCl buffer (pH 8.0) in a filter bag BagPage (Interscience, Paris, France) using a pulsifier equipment. Next, the filtrate which containing spores and the mycelium that grew on the ham slices was treated for the DNA extraction and for the determination of mould counts (cfu/g). The solid non-filtrate substrate that contains dry-cured ham without mycelium and spores was used for mycotoxin extraction.

2.3. Mycotoxins determination

2.3.1. Mycotoxins extraction

The solid non-filtrate was macerated for 1 h on 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. The slurry was then transferred to a separatory funnel and the bottom phase was twice filtered through anhydrous sodium sulphate and Whatman no. 1 filter paper (Whatman, Tokio, Japan). Then, the filtrate was mixed with 50 mL of hexane, shaked for 30 min in the dark and transferred to a separatory 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 pore size nylon membrane (MSI, Westboro, USA), and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4 °C in the dark until required and resuspended in 200 mL of acetonitrile just before high-pressure liquid chromatography–mass spectrometry (HPLC–MS) analysis.

2.3.2. Mycotoxins analysis

Mycotoxins production was analyzed by HPLC–MS according to Sosa et al. (2002) in a Hewlett Packard series 1100 apparatus

(Hewlett Packard, Palo Alto, USA). A Supelcosil LC-18 column (SUPELCO, Bellafonte, USA) was used with mobile phases (A) 100% water and (B) 0.05% trifluoroacetic acid in acetonitrile in a gradient from 10% to 99%. AFB1, OTA and patulin were identified in a Finnigan LCQ Mass Spectrometer (Finnigan, San Jose, USA) with atmospheric pressure chemical ionization source (APCI), according to their retention time and molecular mass. Under described conditions, AFB1, OTA and patulin eluted at 18.5, 21.5 and 7.4 min as a protonated molecular ion of *m/z* 313, 404 and 137, respectively. The calibration curves for AFB1, OTA and patulin by HPLC–MS revealed a linear relationship (r^2 = 0.9871, 0.9901 and 0.9815) between detector response and amount of mycotoxin standards (Sigma–Aldrich Química S.A., Madrid, Spain) from 1 to 100 ng/mL for AFB1 and OTA and from 10 to 100 ng/mL for patulin.

The minimum detectable value or limit of detection (LOD) was estimated from the calibration curve, according to the equation: $\mathrm{LOD} = 3(s_B^2 + s_i^2 + (i/m)^2 s_m)^{1/2}/m$ (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 LODs were 0.079, 1 and 5 μg/kg for AFB1, OTA and patulin, respectively, being the LOQs for the above mycotoxins 0.240, 3 and 15 μg/kg, respectively.

2.3.3. Method validation

The method was initially validated by analysis of replicated samples spiked with AFB1, OTA and patulin ($n = 3$). Samples of 5 g of dry-cured ham slices showing undetectable amounts of AFB1, OTA and patulin were spiked separately with 12.5, 25 and 50 μg/kg of AFB1, OTA and patulin. Spiked samples were allowed to equilibrate for 2 h prior to extraction. Mean recoveries obtained at each spiking level of 12.5, 25 and 50 μg/kg were respectively 92, 95 and 96% for AFB1, 90, 94 and 96% for OTA, and 78, 91 and 90% for patulin.

2.4. Mould growth

2.4.1. Determination of the fungal growth by plate counting

Before DNA extraction, from the filtrate solution 10-fold dilutions were prepared in 0.1% sterile peptone in water. A volume of 100 μL of these solutions was plated out on Potato Dextrose Agar (PDA, Scharlau Chemie S.A., Spain). These plates were incubated at 25 °C for 5 days, the colonies were counted and the number of cfu/g was calculated.

2.4.2. qPCR assays

2.4.2.1. DNA extraction. For DNA extraction, the filtrate obtained within each bag was transferred to a sterile tube, and centrifuged at 13,000 rpm for 10 min. Pellets were resuspended in 100 μL of sterile nanopure water, boiled (95 °C for 15 min) to release the DNA, and cooled on ice for 10 min. Next, 500 μL CTAB buffer (5 g D-sorbitol, 2 g N-lauroylsarcosine, 1.6 g/L CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 2 g PVPP, 0.1 M Tris–HCl, pH 8.0) was added together with 10 μL of a proteinase K solution (10 mg/mL) before incubation at 65 °C for 1 h. Samples were centrifuged at 13,000 rpm for 5 min, and the supernatant was transferred to a new tube with 500 μL chloroform, vortexed, and centrifuged at 13,000 rpm for 20 min. The upper layer was transferred to a new tube and 10 μL RNase solution (10 mg/mL) was added before incubation at 37 °C for 1 h. An equal volume of chloroform was then added, vortexed, and centrifuged at 13,000 rpm for 5 min. Finally, the aqueous phase was processed according to the EZNA Fungal DNA Mini Kit (Omega bio-teck, Doraville, USA) protocol, starting from DNA precipitation by adding 500 μL of isopropanol (step 4 protocol B). In the final step, DNA was eluted in 100 μL of elution buffer pre-warmed to 65 °C and kept at −20 °C until used as template for PCR amplification.

2.4.2.2. Specific qPCR for quantification AFB1, OTA and patulin producers. The qPCR analyses were performed on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). qPCR reactions were prepared in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). The SYBR Green qPCR protocols were carried out in volumes of 25 μL containing 5 μL of template DNA, 12.5 μL of 2× SYBR® *Premix Ex Taq*™ (Takara Bio Inc., Shiga, Japan), 0.5 μL of 50× ROX™ Reference Dye (Takara) and optimal primers concentration of each qPCR reaction (Table 1) according to Rodríguez, Luque et al. (2011), Rodríguez, Rodríguez, Gordillo et al. (2011), Rodríguez, Rodríguez, Luque et al. (2011). 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. All PCR reactions were performed in triplicates and control sample without DNA template were included in all assays. Threshold cycle (Ct) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, was calculated by the 7500 Fast System SDS software (Applied Biosystems). The fungal load by PCR was calculated after replacing of Ct values from the inoculated ham slices in the three different previously standard curves for AFB1, OTA and patulin producing strains from inoculated dry-cured hams (Rodríguez, Luque et al., 2011; Rodríguez, Rodríguez, Gordillo et al., 2011; Rodríguez, Rodríguez, Luque et al., 2011). The limits of detection were 1 log cfu/g in all qPCR used.

2.4.2.3. Control qPCR to detect and quantify β-tubulin gene. To avoid false negative results in the specific quantification of AFB1, OTA and patulin producing moulds, the presence of fungal DNA in all samples was tested by the designed qPCR to detect the universal fungal *β-tubulin* gene. For this, specific primers TubF1 (5′-TCCCTTCGGCAAGCTTTTC-3′) and TubR1 (5′-TGTTACCAGCACCGGACTGA-3′) were designed to target a conserved region of the *β-tubulin* gene using Primer Express software (Applied Biosystems, Foster City, CA, USA). This primer pair (TubF1/TubR1) amplified an amplicon of 62 bp from nucleotide 320 to 363 bp according to the published sequence of the *β-tubulin* gene (accession number JN398145). This qPCR protocol was carried out 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 (Takara) and 1.5 μL of each primer (10 μM). The qPCR was conducted by the thermal cycling conditions described in 2.4.2.2. Section. All PCR reactions were performed in triplicates and control sample without

Table 1
Primers and qPCR conditions used in this study.

Mycotoxin	Primer pair	Primer name	Nucleotide sequences (5′–3′)	Primers concentration (nM)
AFB1	F/R-omt	F-omt	GGCCGCCGCTTTGATC TAGG	200
		R-omt	ACCACGACCGCCGCC	400
OTA	F/R-npstr	F-npstr	GCCGCCCTCTGTCATT CCAAG	400
		R-npstr	GCCATCTCCAAACTCAA GCGTG	400
Patulin	F/R-idhtrb	F-idhtrb	GGCATCCATCATCGT	400
		R-idhtrb	CTGTTCCTCCACCCA	700

DNA template were included in all runs. The Ct value was calculated as the cycle number at which the concentration increase became exponential. The limit of detection was 1 log cfu/g.

2.5. Statistical analysis

All the statistical analyses were performed with the SPSS v.15.0. One way analysis of variance (ANOVA) was carried out to evaluate any significant differences of fungal growth and mycotoxin production at the different incubation times and RH conditions assayed between producing strains of the same mycotoxin. Afterwards, Tukey's test was applied to compare the mean values obtained. Statistical significance was set at $P \leq 0.05$. Linear regressions between mycotoxin levels and arbitrary area units were performed using the REG Procedure of the SPSS v.15.0. The relationships between the fungal growth determined by qPCR and by plating and between the fungal load obtained by qPCR based on specific toxigenic genes and those obtained by qPCR based on *β-tubulin* gene were evaluated by Pearson's correlation coefficients. Standard errors of prediction of above coefficients were also calculated.

3. Results

3.1. Quantification of fungal growth by counting plate and by qPCR

Fungal growth in dry-cured ham slices was determined by plate count throughout incubation time in the two RH values tested (Table 2).

At the end of the incubation period, the highest counts were recorded at 97% RH for AFB1 and patulin producing strains and at 84% RH for OTA producing moulds (Table 2). At this point, at both RH tested, all studied moulds showed counts from 2 to 4 log cfu/g higher than initial inoculums ($P \leq 0.05$) (Table 2).

On the other hand, significant differences in mould growth at 97% RH were observed between the two producing strains of both OTA and patulin. Thus, at the end of incubation *P. nordicum* reached counts around 1.5 log cfu/g higher than *A. ochraceus* (Table 2). In this sense, *P. expansum* showed from 1 to 2 log cfu/g higher than *P. griseofulvum* after 7 days of incubation (Table 2). However, at 84% RH, the two strains tested for each mycotoxin reached similar counts in all cases ($P \leq 0.05$).

The natural fungal contamination of the control samples was lower than 1 log cfu/g after 21 days of incubation and no typical colonies of *A. flavus*, *A. parasiticus*, *P. expansum*, *P. griseofulvum*, *P. nordicum* and *A. ochraceus* spp were found (data not shown).

Growth data determined by qPCR (Fig. 1) were very similar to the above reported counts by plating. No significant differences between counts obtained by these two ways were obtained ($P \leq 0.05$). Moreover, correlation between counts by plating and by qPCR was 0.97, 0.91, and 0.92 for the AFB1, OTA and patulin producing strains, respectively, with a respective standard error of prediction of 0.15, 0.20 and 0.23 log cfu/g. When fungal load obtained by qPCR testing the specific toxigenic genes were compared with counts obtained using the structural fungal *β-tubulin* gene, a high correlation of 0.97, 0.96 and 0.98 with *omt-1*, *otanps*PN and *idh genes*, respectively, with a respective standard error of prediction of 0.21, 0.27 and 0.23 log cfu/g, was also found.

3.2. Production of mycotoxins in dry-cured ham

In negative control from non-inoculated dry-cured ham slices samples, none of the mycotoxins tested were found after 21 days of incubation (data not shown).

3.2.1. Aflatoxin B1

The two aflatoxigenic strains synthesized AFB1 in dry-cured ham slices at the two RH tested (Table 3, Fig. 1a, b). The production by the two species examined was very similar ranging from 0.194 to 0.250 μg/g after 21 days of incubation. However, some differences in the dynamic of AFB1 production between these two strains were observed in both RH. *A. parasiticus* showed a significantly higher production ($P \leq 0.05$) than *A. flavus* at day 14 of incubation. AFB1 was detected in ham inoculated with *A. flavus* after 7 days showing a progressive increase during the incubation. However, *A. parasiticus* produced detectable amounts of AFB1 after 14 days of incubation and a significant decrease of this amount was observed at the end of incubation.

3.2.2. Ochratoxin A

OTA was detected in dry-cured ham slices inoculated with both ochratoxigenic strains at the two RH tested (Table 3, Fig. 1c, d). The production of this mycotoxin ranged from 0.012 to 0.088 μg/g. *P. nordicum* yielded significantly higher amount of OTA than *A. ochraceus* ($P \leq 0.05$) in all sampling times. In addition, *P. nordicum* showed production at day 7 of incubation, while *A. ochraceus* produced detectable amount of OTA after 14 days of incubation. Accumulation of OTA on ham was not progressive throughout incubation period. Thus, a decrease of OTA in the middle of incubation period and a new increase at day 21 of incubation was observed for *P. nordicum* at the two tested RH. *A. ochraceus* showed higher levels of OTA at 14 than 21 days of incubation ($P \leq 0.05$, Table 3) at two evaluated RH. No significant differences in OTA production due to the two RH tested were observed ($P \leq 0.05$), except for *A. ochraceus* at day 14 of incubation, that showed higher levels at 97 than 84% RH.

Table 2
Quantification of fungal growth (log cfu/g) from mycotoxin producing moulds inoculated dry-cured ham by plate counting at different sampling times with 84% and 97% RH (initial inoculum was 3.0 log cfu/g).

Mycotoxin	Mycotoxin producing strains	Average count (log cfu/g)							
		84% RH				97% RH			
		Incubation time (days)				Incubation time (days)			
		0	7	14	21	0	7	14	21
AFB1	*A. flavus*	3.0 ± 0.42^{a}	3.5 ± 0.12^{a}	4.9 ± 0.04^{b}	5.7 ± 0.29^{c}	2.8 ± 0.50^{a}	4.8 ± 0.04^{b}	6.1 ± 0.04^{c}	7.4 ± 0.29^{d}
	A. parasiticus	2.7 ± 0.10^{a}	3.3 ± 0.16^{b}	$4.1 \pm 0.04^{c,d}$	$4.7 \pm 0.08^{d,e}$	$3.7 \pm 0.11^{b,c}$	4.9 ± 0.16^{e}	6.4 ± 0.04^{f}	7.7 ± 0.74^{g}
OTA	*P. nordicum*	3.2 ± 0.28^{a}	4.6 ± 0.18^{b}	5.6 ± 0.24^{c}	7.2 ± 0.58^{e}	2.2 ± 0.80^{a}	4.6 ± 0.20^{b}	$5.4 \pm 0.21^{b,c}$	$6.4 \pm 0.27^{d,2}$
	A. ochraceus	3.0 ± 0.38^{a}	3.9 ± 0.09^{b}	5.6 ± 0.10^{d}	6.8 ± 0.59^{e}	2.9 ± 0.42^{a}	3.8 ± 0.11^{b}	4.8 ± 0.17^{c}	$4.9 \pm 0.14^{c,d,1}$
Patulin	*P. expansum*	2.8 ± 0.15^{a}	3.1 ± 0.15^{a}	4.1 ± 0.04^{b}	5.3 ± 0.11^{c}	3.3 ± 0.39^{a}	$6.8 \pm 0.12^{d,2}$	$7.4 \pm 0.04^{e,2}$	$7.5 \pm 0.07^{e,2}$
	P. griseofulvum	3.0 ± 0.30^{a}	3.0 ± 0.10^{a}	3.9 ± 0.22^{b}	5.1 ± 0.08^{c}	3.1 ± 0.50^{a}	$4.1 \pm 0.17^{b,1}$	$6.3 \pm 0.22^{d,1}$	$6.6 \pm 0.04^{d,1}$

Mean values of both RH along a row with different letters as superscript are significantly different ($P \leq 0.05$) (the two RH were compared at the same time). Mean values with different numbers as superscript along a column for the same mycotoxin are significantly different ($P \leq 0.05$).

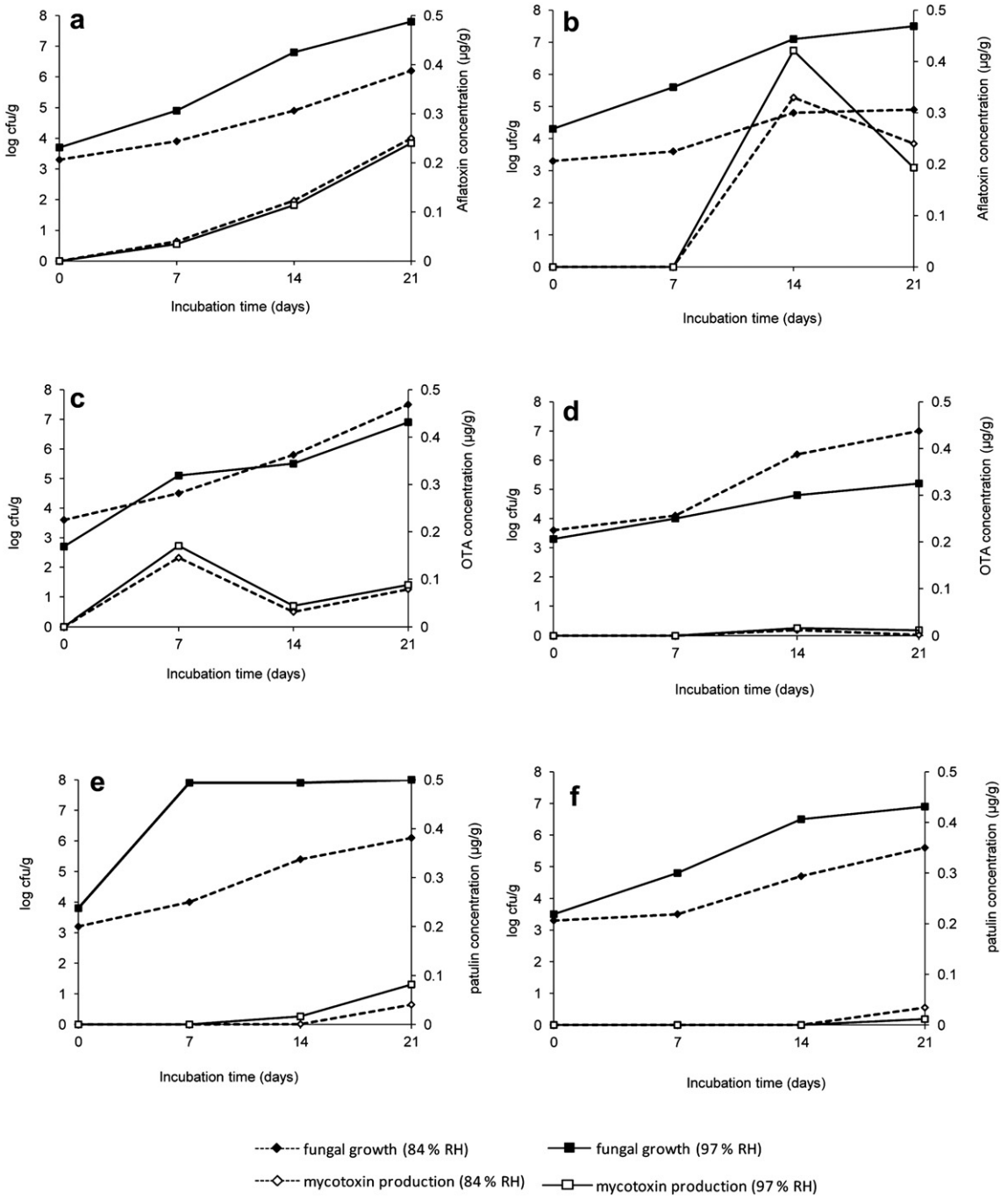

Fig. 1. Evolution of AFB1, OTA or patulin production and the fungal load (log cfu/g) obtained by qPCR of *Aspergillus flavus* (a), *Aspergillus parasiticus* (b), *Penicillium nordicum* (c), *Aspergillus ochraceus* (d), *Penicillium expansum* (e) and *Penicillium griseofulvum* (f) on inoculated dry-cured ham throughout 21 days of incubation at 84% and 97% RH values.

3.2.3. Patulin

Patulin was produced by both species inoculated in the two tested RH, showing *P. expansum* significantly higher yield than *P. griseofulvum* at 97% RH (Table 3, Fig. 1e, f). Accumulation of this mycotoxin on inoculated dry-cured ham slices ranged from 0.034 to 0.082 μg/g after 21 days of incubation. None of the two species evaluated produced patulin at day 7 of incubation and only *P. expansum* produced this mycotoxin at day 14 of incubation and 97% RH. The two species showed significant differences in the production because of RH ($P \leq 0.05$). Thus, the highest yields were recorded at 97% RH for *P. expansum* and at 84% RH for *P. griseofulvum*.

4. Discussion

In this study, the hazard of mycotoxins production in dry-cured ham due to growth of toxigenic mould was evaluated. In addition, the ability of specific qPCR methods to quantify toxigenic moulds before mycotoxins production was also tested. First, the growth of all fungal strains on ham slices at two RH was evaluated. RH showed influence over growth for most of the strains tested. Thus, AFB1 and patulin producing species seemed to reach higher counts at 97% (A_w of hams slices 0.92) than 84% RH (A_w ham slices of 0.84), while ochratoxigenic species reached higher counts at 84% RH. Values of A_w higher than those found could be detected on the

Table 3
Quantification of mycotoxin production (μg/g) on inoculated dry-cured ham slices at different sampling times with 84% and 97% RH (initial inoculum was 3.0 log cfu/g).

Mycotoxin	Mycotoxin producing strains	Mycotoxin production (μg/g)							
		84% RH				97% RH			
		Incubation time (days)				Incubation time (days)			
		0	7	14	21	0	7	14	21
AFB1	*A. flavus*	<LOD^{a}	0.040 ± 0.006^{a,2}	0.123 ± 0.016^{b,1}	0.250 ± 0.024^{c}	<LOD^{a}	0.034 ± 0.002^{a,2}	0.114 ± 0.021^{b,1}	0.240 ± 0.030^{c}
	A. parasiticus	<LOD^{a}	<LOD^{a,1}	0.330 ± 0.015^{d,2}	0.240 ± 0.038^{c}	<LOD^{a}	<LOD^{a,1}	0.421 ± 0.037^{e,2}	0.194 ± 0.059^{b}
OTA	*P. nordicum*	<LOD^{a}	0.145 ± 0.013^{d,2}	0.031 ± 0.007^{b,2}	0.078 ± 0.006^{c,2}	<LOD^{a}	0.170 ± 0.007^{d,2}	0.043 ± 0.022^{b,2}	0.088 ± 0.013^{c,2}
	A. ochraceus	<LOD^{a}	<LOD^{a,1}	0.012 ± 0.004^{b,1}	<LOQ^{a,1}	<LOD^{a}	<LOD^{a,1}	0.016 ± 0.002^{c,1}	0.012 ± 0.006^{b,1}
Patulin	*P. expansum*	<LOD^{a}	<LOD^{a}	<LOD^{a}	0.040 ± 0.003^{c}	<LOD^{a}	<LOD^{a}	0.016 ± 0.009^{b,2}	0.082 ± 0.010^{d,2}
	P. griseofulvum	<LOD^{a}	<LOD^{a}	<LOD^{a}	0.034 ± 0.005^{b}	<LOD^{a}	<LOD^{a}	<LOD^{a,1}	<LOQ^{a,1}

Mean values of both RH along a row with different letters as superscript are significantly different ($P \leq 0.05$) (the two RH were compared at the same time). Mean values with different numbers as superscript along a column for the same mycotoxin are significantly different ($P \leq 0.05$).
LOD: limit of detection.
LOQ: limit of quantification.

surface of the product at the first stages of processing (post-salting), but in this stage temperature around 5 °C does not favour mould growth. However, in drying and cellar stages or during storage of the final products the temperature may be even higher than 25 °C. At this temperature mould growth may be favoured by 0.92 A_w that could be found on the hams at the beginning of drying stage (Andrés et al., 2005; Rodríguez et al., 1994). Previous studies have demonstrated that growth of moulds may occur in a wide range of RH when A_w of the product ranges between 0.81 and 0.99. In these conditions mould growth depends on other variables like pH, atmosphere, solute concentrations or mould species studied (García, Ramos, Sanchis, & Marín, 2011; Gibson, Baranyi, Pitt, Eyles, & Roberts, 1994; Sautour, Dantigny, Divies, & Bensoussan, 2001). It is also assumed at lower RH in dry-cured ham *Aspergillus* species grow better than those of *Penicillium* (Comi, Orlic, Redzepovic, Urso, & Iacumin, 2004; Nuñez et al., 1996). However, in this work, ochratoxigenic strains from the *Penicillium* or the *Aspergillus* genus showed the same behaviour, growing better at 84 than at 97% RH. In contrast the two aflatoxigenic *Aspergillus* grew better at 97 than 84% RH.

Some differences have been also observed among species that produce the same mycotoxin. For OTA and patulin producing species, after 21 days of incubation at 97% RH counts were slightly higher for *P. nordicum* than *A. ochraceus* and for *P. expansum* than *P. griseofulvum*, respectively. In addition, *P. expansum* was the only species tested which did not show a significant lag phase at RH 97%. This fact could be justified by the ability of adaptation of *P. nordicum* to NaCl and protein rich foods as it occur in fermented meats and cheeses (Bogs et al., 2006; Schmidt-Heydt, Graf, Batzler, & Geisen, 2011). On the other hand, the rapid growth of *P. expansum* is justified by the high RH, more appropriated for these species than for *P. griseofulvum* (Baert et al., 2007; Gougouli & Koutsoumanis, 2010).

The hazard of AFB1, OTA and patulin production on ham is demonstrated in the present work. The production of OTA and aflatoxins has been previously reported in dry-cured meat system (Battilani et al., 2010; Rojas et al., 1991). In addition, OTA has been detected in dry-cured ham from pigs fed with contaminated diet of OTA (Dall'Asta et al., 2010). Given that none of the mycotoxins were detected in the non-inoculated ham slices, its presence should not be a consequence of pigs fed with contaminated diets. On the other hand, as far now, production of patulin has not been reported on dry-cured hams, although numerous patulin producing strains have been isolated from this product (Martín et al., 2004). Moreover, in this work the dynamic of production of AFB1, OTA, and patulin in dry-cured ham slices at two different RH is reported. Generally, the production of these toxins on the hams was slightly higher at the greatest RH (97%), except for *P. griseofulvum*. The metabolisms of moulds is highly affected by the A_w of the substrate (which is related to RH of processing), and high values of this parameter are usually favourable for the growth and physiological activity of microorganisms, such as mycotoxins biosynthesis (Asefa et al., 2011; Gqaleni, Smith, Lacey, & Gettinby, 1997; Schmidt-Heydt et al., 2009, 2011; Sosa et al., 2002).

The mycotoxin producing capacity of each mycotoxigenic mould used was also evaluated. AFB1 was the mycotoxin produced in highest amount on inoculated dry-cured ham. These values were higher (two order of magnitude) than those reported by Rojas et al. (1991). These differences found in the AFB1 accumulation in dry-cured ham could be due to the complex mechanism of aflatoxins biosynthesis, which depends on strain used and growth conditions including substrate composition or physical factors such as pH, A_w or temperature (Giorni, Battilani, Pietri, & Magan, 2008; Molina & Giannuzzi, 2002). In fact, Rojas et al. (1991) found differences in AFB1 accumulation on the dry-cured ham system depending of the species tested, substrate, A_w, RH and temperature of incubation. In addition, a different dynamic of AFB1 accumulation for both species throughout incubation was observed. For *A. flavus*, the aflatoxin production was progressive from day 7, while for *A. parasiticus* the accumulation occurred mostly after 14 days of incubation and then a decrease was observed. This reduction could be explained by the reaction of aflatoxin with some meat components such as proteins or free amino acids like lysine (Ashoor & Chu, 1975). As results, it can be form some abducts with a different mass spectrum (Sabbioni, 1990; Xu, Qian, Tang, Su, & Wang, 2010) which cannot be identified as AFB1 by HPLC–MS. These reactions could be favoured by the higher amount of AFB1 produced by *A. parasiticus*. Although is not established a legal limit for AFB1 in dry-cured ham, values observed for this mycotoxin in only 21 days of incubation are in some cases above of the legal limit of 2–5 μg/kg reported for other foods, such as cereals or spices (Commission of the European Communities, 2010).

OTA was produced in significantly lower amounts than AFB1. However, the concentration of OTA were upper than those reported by Dall'Asta et al. (2010) in dry-cured ham from pigs fed with contaminated diet, but lower than those cited by Battilani et al. (2010) in a dry-cured meat model system. These differences could be explained by the variability on mycotoxin production among producing strains, and the influence of environmental factors in the OTA biosynthesis (Khalesi & Khatib, 2011). The dynamics of OTA production of both species throughout the incubation was similar. However, the accumulation occurred mostly at day 7 for *P. nordicum* and at day 14 for *A. ochraceus*. These changes in the OTA accumulation in dry-cured ham and other foods have been reported by several authors (Alborch, Bragulat, Abarca, & Cabañes, 2011; Sonjak, Ličen, Frisvad, & Gunde-Cimerman, 2011). In fact, OTA rapidly decrease after direct contamination of dry-cured ham (Bailly et al.,

2005). This decrease has been attributed to the reaction of OTA with some amino acids to form abducts (Bailly et al., 2005) in a similar way to that described for penicillic acid (Ciegler, Mintzlaff, Weisleder, & Leistner, 1972) or aflatoxins (Ashoor & Chu, 1975). Despite of this amount of OTA on dry-cured ham slices after the 21 days of incubation is higher that the legal limit for pork meat and derived products (1 μg/kg) established in the Italian legislation (Ministero della Sanità, 1999).

Patulin producing moulds yielded lower quantities of toxin on inoculated ham than the other mycotoxigenic moulds studied. In this sense, Bailly et al. (2005) reported that a strain of *Penicillium patulum* was unable to produce any detectable amount of patulin in dry meat, whereas it produced a high quantity in YES culture medium. This fact could be related to the ability of patulin to bind with sulfhydryl compounds which leading to rapid disappearance of the toxin that cannot be recovered from these adducts (Fliege & Metzler, 1999; Lieu & Bullerman, 1978). Despite the relatively lower amount of patulin found in dry-cured ham slices inoculated with *P. expansum*, it is above of legal limit of 25–50 μg/kg established for other foods such as fruits or apple products (Commission of the European Communities, 2006).

All the tested strains have demonstrated their ability to produce mycotoxins in dry-cured ham and at conditions of RH that could be found on the hams in drying and cellar stages and also during storage. Given the amount detected in only 21 days above the legal limits, it should be assumed that growth of mycotoxigenic moulds on dry-cured ham may suppose a potential hazard for the consumers. Furthermore, it should be considered that dry-cured hams are aged for several months (between 6 and 24) at 15–25 °C with potentially toxigenic moulds on their surface (Asefa et al., 2009; Núñez et al., 1996; Rojas et al., 1991; Tabuc, Bailly, Bailly, Querin, & Guerre, 2004). During this time, some of the metabolites could be excreted to the ham and their possible presence should be taken into consideration, especially because dry-cured ham is frequently heavily covered by fungal mycelium. This potential should be carefully evaluated when assessing the risk posed by the presence of these species on dry-cured meats. However, it should be keep in mind that contrary to the design of this study, individual moulds do not grow as isolated entities on cured ham in production facilities but rather grow in competition with each other and with yeasts and bacteria. This interaction may reduce the production of metabolites or these compounds might be metabolized by other microorganisms. Thus, many lactic acid bacteria isolated from foods can produce a variety of compounds with antifungal activity such as organic acids, reuterin, fatty acids, or proteins, and some of them have the potential to reduce mycotoxins (Dalié, Deschamps, & Richard-Forget, 2010; Rouse, Harnett, Vaughan, & van Sinderen, 2008). Also, it has been described that strains of *Debaryomyces hansenii* produce killer proteins that inhibit mould growth in milk products (Liu & Tsao, 2009). In addition, several yeasts, including *D. hansenii*, are able to reduce the accumulation of OTA through regulation of its biosynthesis at transcriptional level, adsorption to yeast cell wall or toxin degradation (Gil-Serna, Patiño, Cortés, González-Jaén, & Vázquez, 2011; Patharajan et al., 2011). Given that some of these microorganisms are commonly found on dry-cured ham, further studies is needed to know their influence on mycotoxins accumulation, and also how mycotoxins penetrate to inner tissues of the dry-cured hams. On the other hand, the amount of mycotoxin seemed to decrease through the incubation time, likely due to the formation of abducts with meat constituent, and the toxicological signification of this conversion should be elucidated.

To date, most of studies about mycotoxins in foods are only focused on evaluation of production in different incubation conditions (Battilani et al., 2010; Sant'Ana et al., 2010). However, for preventive food safety programs it could be of great interest to detect and quantify toxigenic moulds on the hams before mycotoxins are produced. The qPCR methods could be used to estimate the growth of mycotoxigenic moulds on the dry-cured hams. In this study, high correlations between counts obtained by qPCR assays designed on the basis of genes involved in the aflatoxin, OTA and patulin biosynthesis (Rodríguez, Luque et al., 2011; Rodríguez, Rodríguez, Gordillo et al., 2011; Rodríguez, Rodríguez, Luque et al., 2011) and those obtained by plating were observed in all cases. Similar results have been reported in quantification of *A. flavus* by Mayer et al. (2003) and Passone et al. (2010). Thus, these methods may be good indicators for predicting the presence of these toxigenic moulds in dry-cured ham. Furthermore, the high correlation observed between quantification by toxigenic genes and by the structural fungal gene *β-tubulin* confirmed the suitability of qPCR based on these toxin biosynthesis genes to quantify these mycotoxins producing moulds.

All the assayed qPCR methods revealed the growth of mycotoxin producing moulds before AFB1, OTA or patulin was detected. Since the assayed qPCR methods quantify toxigenic moulds, it could be appropriated to establish a tentative fungal level on the hams over which there is a hazard of mycotoxins contamination. From the results obtained in this work, with levels of 3.5, 4.6 and 4.1 log cfu/g of AFB1, OTA and patulin producing moulds, respectively, these toxins were detected in ham. Thus, to protect the consumer health level of toxigenic moulds in the product with minimal risk of mycotoxins accumulation could be tentatively established at 3 log cfu/g. This limit could be of great interest for HACCP programs and qPCR may be very useful for monitoring toxigenic moulds. In this way, quantification of aflatoxin, OTA and patulin producing moulds allow a reliable and rapid estimation of the contamination with these moulds for evaluating the quality of raw materials/ingredients, and monitoring of hygienic control in dry-cured ham. Results obtained after monitoring may allow taking rapid corrective actions to avoid mycotoxins accumulation in the final product.

In conclusion, the tested mycotoxigenic strains are able to grow and produce AFB1, OTA or patulin on dry-cured hams slices at different RH, and can be a potential hazard for consumers that should be carefully evaluated. The specific qPCR assays to quantify aflatoxin, OTA and patulin producing moulds would contribute to food safety improving the prediction of the potential production of cited toxins in dry-cured ham before they can be detected, thereby the risk of contamination of dry-cured ham would be minimized.

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