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Development of real-time PCR methods to quantify patulin-producing molds in food products

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ABSTRACT

Patulin is a mycotoxin produced by different *Penicillium* and *Aspergillus* strains isolated from food products. To improve food safety, the presence of patulin-producing molds in foods should be quantified. In the present work, two real-time (RTi) PCR protocols based on SYBR Green and TaqMan were developed. Thirty four patulin producers and 28 non-producers strains belonging to different species usually reported in food products were used. The patulin production was tested by mycellar electrokinetic capillary electrophoresis (MECE) and high-pressure liquid chromatography-mass spectrometry (HPLC-MS). A primer pair F-idhtrb/R-idhtrb and the probe *IDHprobe* were designed from the isoepoxydon dehydrogenase (*idh*) gene, involved in patulin biosynthesis. The functionality of the developed method was demonstrated by the high linear relationship of the standard curves constructed with the *idh* gene copy number and Ct values for the different patulin producers tested. The ability to quantify patulin producers of the developed SYBR Green and TaqMan assays in artificially inoculated food samples was successful, with a minimum threshold of 10 conidia g^{-1} per reaction. The developed methods quantified with high efficiency fungal load in foods. These RTi-PCR protocols, are proposed to be used to quantify patulin-producing molds in food products and to prevent patulin from entering the food chain.

1. Introduction

Mycotoxigenic molds have been reported among the main sources of contamination in foods (Sweeney and Dobson, 1998), especially in dry-ripened foods. The environmental conditions found in meat and cheeses throughout the ripening process favor growth of a mold population composed mainly by *Penicillium* and *Aspergillus* spp. (Núñez et al., 1996; Kure et al., 2004). Several strains of both genera isolated from these products have been reported as patulin producers (Erdogan et al., 2003; Erdogan and Sert, 2004; Martín et al., 2004).

Patulin is a tetraketide mycotoxin produced by a variety of different fungi, most of them from *Penicillium* genus, such as *P. expansum* and *P. griseofulvum* (Niessen, 2007), but also from *Aspergillus* and *Emericella* spp. (Selmanoglu and Koçkaya, 2004; Labuda and Tančinová, 2006; Selmanoglu, 2006). To improve food safety and to protect consumers from hazardous mycotoxins, food products should be screened for molds with patulin-producing potential at critical control points.

Real-time quantitative PCR (RTi-PCR) provides a tool for accurate and sensitive quantification of target DNA (Gil-Serna et al., 2009) that could be applied to detect patulin-producing molds. To develop specific RTi-PCR assays for detecting patulin-producing molds, it is essential to use sensitive methods to select a collection of reference strains for both patulin producers and non-producers. Unlike some other mycotoxins, there is only limited information on patulin production from molds, even from culture collections. Micellar electrokinetic capillary electrophoresis (MECE) and high-pressure liquid chromatography-mass spectrometry (HPLC-MS), have been reported as sensitive methods to detect patulin produced by molds commonly found in foods (Tsao and Zhou, 2000; Martín et al., 2004), being able to be used to assure a collection of patulin-producing and non-producing strains.

Several RTi-PCR assays have been reported for detection of toxigenic molds using DNA-associating dyes (SYBR Green) and fluorescently labeled oligoprobes (TaqMan) (Atoui et al., 2007; Fredlund et al., 2008; Selma et al., 2008; Nicolaisen et al., 2009; Suanthie et al., 2009). However, no RTi-PCR protocol has yet been developed to quantify patulin-producing molds in foods. The lower cost of RTi-PCR based on SYBR Green is an advantage for routine analyses of food commodities. However, the SYBR Green reagent system may lose specificity if primers–dimers are formed or

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nonspecific fragments are present (Kubista et al., 2006), whereas TaqMan oligoprobes may offer a more accurate detection.

In both SYBR Green and TaqMan RTi-PCR, the target sequence to design primers is essential for a good power of discrimination and sensitivity of the method. The gene cluster involved in patulin biosynthesis has recently been isolated and only some genes related to patulin biosynthesis have been identified. The isoepoxydon dehydrogenase (*idh*) gene in *Penicillium* and *Aspergillus* spp. (Paterson, 2004) codes for a key enzyme of the patulin biosynthesis in all producer molds (Puel et al., 2007, 2010). In addition, this gene has been used as target to design primers IDH1 and IDH2 for the detection of patulin-producing molds by conventional PCR (Paterson et al., 2000; Dombrink-Kurtzman, 2007). Thus, the *idh* gene may be used to design primers and probes for RTi-PCR protocols.

The efficiency of PCR quantification methods can be seriously affected by the presence of inhibitors in the food matrix, such as proteinases (Powell et al., 1994) and other compounds naturally present in foods (Mulé et al., 2006), or by the heterogeneous composition of some food matrices such as cooked minced-meats (Rossmanith et al., 2007). For this reason, testing directly on such foods is required to evaluate the potential application of these methods for quantification of molds. The aim of the present work was to develop sensitive and specific RTi-PCR tests to quantify the main patulin-producing molds in foods.

2. Material and methods

2.1. Fungal strains

Sixty two mold strains, belonging to different species, were obtained from the Centraalbureau voor Schimmelcultures in The Netherlands (CBS), the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark (IBT), the Spanish Type Culture Collection (CECT) and the Culture Collection of Food Hygiene from University of Extremadura. Only 12 of them were known as patulin producers, whereas no information on patulin production was available for the remaining strains, as indicated in Table 1.

2.2. Patulin production

Production of patulin was tested after growing each mold strain in 3-points per plate on Malt Extract Agar (2% malt extract, 2% glucose, 0.1% peptone, and 2% agar) and incubated for 15 days at 25 °C. The content of three petri dishes was extracted with chloroform, filtered twice through anhydrous sodium sulfate and evaporated in a rotatory evaporator at 40 °C as described by Sosa et al. (2002). The residue was resuspended in 5 mL of chloroform, filtered through a 0.45-mm pore size nylon membrane, 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 μL of acetonitrile just before analysis. MECE analysis was carried out according to Martín et al. (2004) in a Beckman P/ACE 5500 model with a photodiode array detector (Beckman Instruments, Fullerton, USA). A fused silica capillary of 57 cm length and 75 μm inside diameter was used for separation with 25 mM sodium tetraborate and 50 mM SDS (pH 9) as running buffer, at 15 kV, maximum current at 200 mA, and a capillary cassette temperature of 23 °C. The absorbance was recorded at 200 and 280 nm wavelengths. For each peak, a spectrum of absorbance between 190 and 600 nm was obtained in the photodiode array detector. HPLC-MS analysis was performed 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% B. Patulin was 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. In both methods, patulin (Sigma Chemical Co., St. Louis, Mo., USA) was used as standard, and the detection limit was 4 μg L^{-1}.

2.3. DNA extraction

Every mold strain was 3-point inoculated on Malt Extract Agar and incubated for 4 days at 25 °C. Mycelium of each strain was scraped off the agar and used for genomic DNA extraction. For this, 50 mg of mycelium was digested with proteinase K and lyticase, frozen in liquid nitrogen, and grinded with mortar and pestle, prior to DNA extraction in a semiautomatic vacuum system, following a method developed for various species of *Penicillium* and *Aspergillus* by Sánchez et al. (2008). The extracted fungal DNA showed absorbance ratios ranging from 1.7 to 1.8 at 260:280 nm.

2.4. Primers and probes design

Genomic DNA from both patulin-producing and non-producing strains was used with primers IDH1 and IDH2, based upon the *idh* gene, for amplification by a conventional PCR method described by Paterson (2004). An amplicon of 600 bp was obtained from most patulin-producing strains of *Penicillium*, *Aspergillus*, and *Emericella* spp. This amplicon was purified, sequenced, and analyzed. Two specific primer pairs (F-idhtrb/R-idhtrb and IDH1/R-idhtra, Table 2) from conserved regions of 600-bp amplicon were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). With these primer pairs, two amplicons of 229 and 162 bp, were respectively obtained in the SYBR Green assay. For evaluating the specificity of the above amplicons, they were aligned with the published sequence of the *idh* gene (GenBank accession no. AF006680) using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), as indicated in Table 2.

In addition, the *IDHprobe* TaqMan probe was designed from the amplicon of 229 bp for the TaqMan assay (Table 2). The above probe was labeled at the 5′ end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher 6-carboxytetramethyl-rodamine (TAMRA).

2.5. RTi-PCR reactions

The Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems) was used for RTi-PCR amplification and detection. RTi-PCR was prepared in triplicates 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 the runs.

2.5.1. SYBR Green RTi-PCR conditions

To select the most efficient primers, F-idhtrb/R-idhtrb and IDH1/R-idhtra primer pairs were first evaluated in the SYBR Green protocol. To optimize the amount of primers in the reactions, patulin-producing strains *Penicillium dipodomyicola* IBT 26223 and *P. griseofulvum* CBS 48584 were used. The primers concentrations tested ranged from 700 to 200 nM. The selected primers F-idhtrb and R-idhtrb were used in the SYBR Green RTi-PCR method. This optimized protocol was carried out in a final volume of 25 μL, containing 2.5 μL of template DNA, 12.5 μL of 2x SYBR® Green PCR Master Mix (Applied Biosystems), and 400 nM and 700 nM of each forward and reverse specific primers, respectively.

Table 1
Patulin production and RTi-PCR data with the specific primers (F-idhtrb and R-idhtrb) and *IDH*probe of the reference mold strains.

Species designation	Strain reference	Information provided by Culture Collections	Patulin production as detected by:	RTi-PCR reactions		
			MECE/HPLC-MS	SYBR Green reaction mean		TaqMan mean $C_t \pm$ SD
				$C_t \pm$ SD[g]	Tm (°C)[h]	
Aspergillus awamori	CBS 101702[a]	Unknown	ND[e]	34.3 ± 0.38	78.5	39.1 ± 0.12
Aspergillus flavus	CECT 2684[b]	Unknown	+[f]	30.8 ± 0.41	87.0	27.9 ± 0.43
Aspergillus flavus	CECT 2687	Unknown	ND	34.0 ± 0.21	72.5	40.0 ± 0.01
Aspergillus flavus	IBT 3696[c]	Unknown	ND	39.9 ± 0.17	71.6	40.0 ± 0.01
Aspergillus flavus	CBS 573.65	Unknown	ND	34.6 ± 0.14	76.5	40.0 ± 0.01
Aspergillus flavus	CBS 120.62	Unknown	ND	34.9 ± 0.27	72.0	40.0 ± 0.01
Aspergillus foetidus	CBS 101708	Unknown	ND	33.3 ± 0.22	70.4	40.0 ± 0.01
Aspergillus fumigatus	CBS 192.65	Unknown	ND	35.5 ± 0.17	71.4	40.0 ± 0.01
Aspergillus niger	CECT 20157	Unknown	ND	36.2 ± 0.01	72.0	40.0 ± 0.01
Aspergillus ochraceoroseus	CBS 101887	Unknown	ND	34.2 ± 0.42	72.1	40.0 ± 0.01
Aspergillus ochraceus	CBS 589.68	Unknown	ND	35.0 ± 0.46	70.4	40.0 ± 0.01
Aspergillus oryzae	CECT 2095	Unknown	+	26.9 ± 0.23	88.4	32.5 ± 0.06
Aspergillus oryzae	CECT 20168	Unknown	+	29.7 ± 0.44	88.1	32.1 ± 0.43
Aspergillus oryzae	CECT 2094	Unknown	ND	38.3 ± 0.28	66.6	40.0 ± 0.01
Aspergillus parasiticus	CECT 2688	Unknown	ND	31.6 ± 0.21	70.4	40.0 ± 0.01
Aspergillus parasiticus	CECT 2682	Unknown	ND	30.9 ± 0.15	82.7	40.0 ± 0.01
Aspergillus parasiticus	CECT 2681	Unknown	ND	38.0 ± 0.25	71.6	40.0 ± 0.01
Aspergillus parasiticus	CBS 571.65	Unknown	ND	38.2 ± 0.23	72.6	40.0 ± 0.01
Aspergillus tamarii	CBS 575.65	Unknown	+	26.9 ± 0.39	88.1	29.0 ± 0.48
Aspergillus tamarii	CBS 109.63	Unknown	+	27.1 ± 0.21	88.1	36.5 ± 0.48
Aspergillus terreus	CBS 601.65	Producer	+	19.4 ± 0.19	88.1	34.3 ± 0.36
Aspergillus toxicarius	CBS 822.72	Unknown	+	17.7 ± 0.15	88.1	27.8 ± 0.21
Aspergillus tubingensis	CECT 20543	Unknown	ND	33.3 ± 0.37	71.6	37.7 ± 0.42
Aspergillus tubingensis	CECT 20545	Unknown	ND	34.3 ± 0.14	71.1	40.0 ± 0.01
Aspergillus versicolor	CECT 2664	Unknown	ND	29.6 ± 0.23	71.0	38.8 ± 0.52
Aspergillus versicolor	CECT 2903	Unknown	ND	28.2 ± 0.44	72.6	40.0 ± 0.01
Aspergillus versicolor	CECT 2814	Unknown	ND	37.2 ± 0.23	80.5	40.0 ± 0.01
Emericella heterothallica	CBS 488.65	Unknown	ND	34.8 ± 0.27	72.6	38.7 ± 0.23
Emericella nidulans	CBS 465.65	Unknown	ND	34.7 ± 0.15	71.2	38.0 ± 0.43
Emericella quadrilineata	CBS 235.65	Unknown	+	18.9 ± 0.40	88.1	19.9 ± 0.03
Emericella rugulosa	CBS 133.60	Unknown	+	28.7 ± 0.25	88.1	27.1 ± 0.24
Emericella variecolor var. Astellana	CBS 133.55	Unknown	+	17.0 ± 0.09	88.1	19.6 ± 0.13
Penicillium aurantiogriseum	CECT 2918	Unknown	+	21.4 ± 0.22	87.8	26.8 ± 0.05
Penicillium aurantiogriseum	CBS 112021	Unknown	+	25.8 ± 0.41	88.1	28.7 ± 0.26
Penicillium aurantiogriseum	CECT 2264	Unknown	ND	31.7 ± 0.02	70.2	40.0 ± 0.01
Penicillium camemberti	CECT 2267	Unknown	+	17.0 ± 0.20	86.9	18.6 ± 0.01
Penicillium camemberti	Pc 1[d]	Unknown	+	21.0 ± 0.19	88.3	15.7 ± 0.07
Penicillium camemberti	CBS 273.97	Unknown	+	25.2 ± 0.32	88.1	24.4 ± 0.09
Penicillium carneum	CBS 468.95	Producer	+	18.7 ± 0.15	88.3	22.0 ± 0.12
Penicillium commune	CBS 311.48	Unknown	+	16.8 ± 0.21	88.1	18.8 ± 0.27
Penicillium commune	CBS 341.59	Unknown	+	15.6 ± 0.25	88.4	17.1 ± 0.21
Penicillium dipodomyicola	IBT 26223	Producer	+	15.8 ± 0.09	86.9	16.7 ± 0.28
Penicillium dipodomyicola	CBS 110425	Producer	+	17.9 ± 0.13	88.1	19.2 ± 0.18
Penicillium dipodomyicola	CBS 110426	Unknown	+	16.3 ± 0.07	88.5	19.1 ± 0.01
Penicillium expansum	CECT 2278	Producer	+	17.6 ± 0.06	86.9	18.3 ± 0.27
Penicillium expansum	CECT 20140	Producer	+	23.6 ± 0.24	88.1	18.1 ± 0.04
Penicillium expansum	CECT 2279	Producer	+	19.1 ± 0.37	88.0	23.6 ± 0.15
Penicillium expansum	CECT 2280	Producer	+	18.2 ± 0.48	88.0	24.6 ± 0.12
Penicillium griseofulvum	IBT 14319	Producer	+	26.5 ± 0.15	88.0	29.5 ± 0.12
Penicillium griseofulvum	CBS 485.84	Producer	+	18.7 ± 0.29	86.9	18.7 ± 0.10
Penicillium griseofulvum	CBS 110420	Producer	+	17.8 ± 0.07	88.1	18.5 ± 0.23
Penicillium griseofulvum	CECT 2919	Producer	+	20.5 ± 0.33	86.9	17.7 ± 0.09
Penicillium melanoconidium	CBS 64195	Unknown	+	27.6 ± 0.32	86.9	32.5 ± 0.42
Penicillium melanoconidium	CBS 109605	Unknown	+	23.8 ± 0.15	88.1	32.2 ± 0.67
Penicillium nordicum	CBS 110769	Unknown	ND	35.0 ± 0.16	66.0	40.0 ± 0.01
Penicillium polonicum	Pc 10	Unknown	+	21.8 ± 0.04	88.1	22.6 ± 0.23
Penicillium polonicum	CBS 112490	Unknown	+	18.8 ± 0.25	88.4	23.6 ± 0.22
Penicillium verrucosum	Pc 4	Unknown	+	22.8 ± 0.16	88.0	32.6 ± 0.23
Penicillium verrucosum	CECT 2906	Unknown	ND	34.8 ± 0.34	71.5	36.6 ± 0.21
Penicillium verrucosum	CBS 323.92	Unknown	ND	35.0 ± 0.23	79.2	40.0 ± 0.01
Penicillium viridicatum	CECT 2320	Unknown	ND	28.8 ± 0.28	70.8	37.6 ± 0.37
Rhizopus oryzae	CBS 607.68	Unknown	ND	31.5 ± 0.35	82.4	40.0 ± 0.01

Patulin-producing strains are shaded.
[a] CBS: Centraalbureau voor Schimmelcultures (The Netherlands).
[b] CECT: Spanish Type Culture Collection.
[c] IBT: Type Culture Collection of the Department of Biotechnology (Technical University of Denmark).
[d] Pc: Culture Collection of Food Hygiene from University of Extremadura (Spain).
[e] ND: patulin production was not detected by MECE or HPLC-MS.
[f] +: patulin production was detected by MECE or HPLC-MS.
[g] Data represent the mean threshold cycle (Ct) ± standard deviation (SD) of the 3 independent experiments each consisting of triplicate samples.
[h] Tm: melting temperature.

Table 2
Nucleotide sequence of primers and dual-labeled probes used for either SYBR Green and TaqMan Real-Time PCR assays.

Primer name	Nucleotide sequences (5′-3′)	Product size	Position[a]	Reference
F-idhtrb	GGCATCCATCATCGT	229 bp	2553	This study
R-idhtrb	CTGTTCCTCCACCCA		2766	This study
IDH1	CAATGTGTCGTACTGTGCCC	162 bp	2135	Paterson (2004)
R-idhtra	GCGTCTTTGCTAGCAACATC		2297	This study
IDHprobe	[FAM]-CCGAAGGGCATCCG-[TAMRA]		2677	This study

[a] Positions according to published sequences of *idh* gene of *Penicillium urticae* (GeneBank accession no. AF006680).

SYBR Green RTi-PCR protocol was conducted with the following thermal cycling conditions: a single step of 10 min at 95 °C, 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. Threshold cycle (Ct) values represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred for each amplification plot. All mold strains used in this study were tested by triplicate with the above method. The size of PCR products was verified by electrophoresis in 2.5% agarose gels and visualized with UV transillumination after ethidium bromide staining. The amplicon was also purified and sequenced to verify if its sequence was from the *idh* gene.

2.5.2. TaqMan RTi-PCR conditions

The selected F-idhtrb/R-idhtrb primer pair and the *IDHprobe* were assayed with the patulin producing strain *P. griseofulvum* CBS 48584 for the TaqMan-based PCR. To optimize the reactions, several concentrations ranging from 700 nM to 200 nM for primers, from 450 nM to 100 nM for probe were assayed. In addition, reaction was performed with and without 0.5 μL of AmpErase uracil-*N*-glycosylase (1u/μL). The reaction mixture for this assay consisted of 12.5 μL of TaqMan® Fast Reagents Starter Kit (Applied Biosystems), 450 nM of each F-idhtrb and R-idhtrb primers, 450 nM of the *IDHprobe*, and 2.5 μL of template DNA in a final volume of 25 μL. After an incubation of 2 min at 50 °C to allow for uracil-*N*-glycosylase (UNG) cleavage, AmpliTaq Gold polymerase was activated by an incubation step for 10 min at 95 °C. All 40 cycles were performed according to the following temperature regime: 95 °C for 15 s and 60 °C for 1 min. Ct determinations were automatically performed by the instrument using default parameters. All mold strains used in this study were tested in triplicate. The size of PCR products was verified by 2.5% gel electrophoresis and visualized with UV transillumination after ethidium bromide staining. The amplicon was also purified and sequenced to verify that the sequence was from the *idh* gene.

2.6. Standard curves

Standard curves were obtained for five patulin-producing strains (*P. expansum* CECT 2278, *P. griseofulvum* IBT 14319, *Emericella rugulosa* CBS 133.60, *Aspergillus tamarii* CBS 109.63, *Aspergillus toxicarius* CBS 822.72), following a procedure described by Mayer et al. (2003). For this, the 600 bp fragment of the *idh* gene amplified with the primers IDH1 and IDH2 (Paterson, 2004) was obtained by conventional PCR as standard stock solution as described above. The concentration of the PCR products in the stock solution was determined in a Biophotometer Eppendorf (Eppendorf AG, Hamburg, Germany) and the number of copies was calculated. Decimal dilutions from stock solution with an initial concentration of 10^8 copies of *idh* gene were used as standard to setup each RTi-PCR reaction. Each standard curve for the five patulin-producing strains was carried out by triplicate. The concentration of unknown samples was calculated by the 7500 Fast System software (Applied Biosystems). The criteria considered for reliability of the designed methods were the correlation coefficient and the amplification efficiency calculated from the formula $E = 10^{-1/S} - 1$ (S being the slope of the linear fit).

2.7. Specificity of RTi-PCR reactions

The specificity of the primer pair F-idhtrb/R-idhtrb was tested on fixed amounts of genomic DNA (0.5 ng for SYBR Green and 1.0 ng for TaqMan assays) from 62 strains of patulin-producing and non-producing *Aspergillus*, *Emericella*, *Penicillium* and *Rhizopus* species commonly found on foods (Table 1). To evaluate the specificity of the primers designed for the SYBR Green assay, the melting temperature (Tm) was automatically calculated and compared with that deduced from the sequence of the expected fragment. In addition, for both SYBR Green and TaqMan assays, the size of amplicons was estimated by electrophoresis in 2.5% agarose gels and was sequenced to verify that the sequence was from the *idh* gene.

2.8. Sensitivity of RTi-PCR on artificially inoculated food matrices

The sensitivity of the optimized RTi-PCR methods was assayed with the DNA extracted from three different type food products, each inoculated with spores of patulin-producing strains. Refrigerated non-sterile commercial cooked ham, an emulsion-type dry-fermented sausage ("salchichón"), and peach were inoculated separately with spores of *P. expansum* CECT 2278 and *Aspergillus flavus* CECT 2684. In addition, cooked ham was also inoculated with *Penicillium commune* CBS 311.48. The spores were extracted flooding 3 plates (20 days old) of Malt Extract Agar with 5 mL of sterile nanopure water containing 10% glycerol (Scharlau Chemie S.A., Spain), and rubbing the surface with a glass rod. The conidial suspension was filtered through Whatman paper No 1, diluted in sterile nanopure water, as necessary, and quantified by microscopy, using a Neubauer counting chamber. Aliquots of 1 mL of 10^2, 10^3, 10^4, 10^5, and 10^6 conidia mL^{-1} of each mold strain were immediately used to inoculate the previously weighted food samples, until final levels of 1, 10, 10^2, 10^3, and 10^4 conidia g^{-1}.

For DNA extraction, 5 g of inoculated food samples were homogenized with 10 mL of Tris–HCl buffer (pH 8.0) in a filter bag BagPage (Interscience, Paris, France) using a pulsifier equipment (Microgen bioproducts, Surrey, UK). The filtrate obtained 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 10 min) to release the DNA, and cooled on ice for 10 min. Next, 500 μL CTAB buffer (20 g L^{-1} CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 0.1 M Tris–HCl, pH 8.0) was added together with 10 μL of a proteinase K solution (10 mg mL^{-1}) 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^{-1}) 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 bioteck, 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.

For RTi-PCR amplification, 3 replicates of 5 μL DNA extracted from the inoculated foods were assayed per run. A negative control from non-inoculated food was included in each experiment. Standard curves were generated for each group of food products.

To assess the absence of PCR inhibitors in the food matrices, five independent genomic DNA extractions from the patulin-producing mold *P. expansum* CECT 2278 were spiked with an equal amount of DNA extracted from non-inoculated food samples. Amplification plots were compared with those obtained by amplifying the same DNA extracted spiked with an equal amount of deionized water.

2.9. Conidia quantification in foods by RTi-PCR

Fungal spores of different patulin-producing strains were inoculated in nine different foods, including cooked meat products (mortadella, cooked turkey breast, and cooked ham), dry-cured meat products (dry-cured ham, the emulsion-type dry-fermented sausage "salchichón", and a minced-meat dry-fermented sausage "chorizo"), and fruits (apple, peach, and pear). Inoculations were carried out according to the most common niche as follows: cooked meat products with *P. commune* CBS 311.48, dry-cured meat products with *A. flavus* CECT 2684, and fruits with *P. expansum* CECT 2278. Three independent tests were run at two levels of fungal spores: low (3×10 conidia g^{-1}) and high (3×10^3 conidia g^{-1}). Reactions were carried out using 2.5 μL of DNA extracted in triplicate. The amount of conidia estimated by RTi-PCR was obtained from standard curves from inoculated foods. A negative control from non-inoculated food was included in each experiment.

2.10. Quantification of fungal growth in inoculated foods

Slices of non-sterile dry-cured ham and apple cut at a surface of 25 cm^2 (approx. 5 g) were placed separately in pre-sterilised orthogonal receptacles made of methacrylate, where the humidity was kept constant at 97% by a saturated KCl solution placed at a reservoir. The slices were inoculated on the surface with spores of the patulin-producing strain *P. expansum* CECT 2278 at a level of 10^3 conidia g^{-1}. Each food slice was incubated by triplicate at 25 °C and sampled at 0, 5, 7, and 10 days of incubation. Negative controls from non-inoculated dry-cured ham and apple slices were analyzed. DNA was extracted as described in Section 2.8 and subjected to the RTi-PCR methods designed as described in Section 2.5. The Ct values obtained with SYBR Green and TaqMan assays for the inoculated samples were used to estimate both the conidial load and the *idh* gene copy number from the corresponding standard curves.

To directly estimate the load of the inoculated mold, the samples were decimally diluted in 0.1% peptone water and spread-plated on Potato Dextrose Agar (Sharlau Chemie S.A., Spain). Colonies showing the characteristic morphology of *P. expansum* after incubation at 25 °C for 4 days were enumerated.

2.11. Statistical analysis

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 mean values. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Production of patulin by MECE and HPLC-MS

MECE and HPLC-MS analyses confirmed patulin production in all 12 strains identified as patulin producers by the culture collections (Table 1). In addition, 22 strains with no information on patulin production in the Culture Collections produced patulin, whereas the 28 remaining strains did not produce detectable amounts by MECE and HPLC-MS.

3.2. Optimization of RTi-PCR conditions

The analysis of the melting curves in the SYBR Green methodology with the two primer pairs designed, IDH1/R-idhtra and F-idhtrb/R-idhtrb, showed just one amplified product, being the Tm values 81.1 ± 0.3 °C and 88.0 ± 0.4 °C, respectively. All Ct values obtained with primer pair F-idhtrb/R-idhtrb were lower than those obtained with IDH1/R-idhtra for the conditions assayed. Thus, the former primer pair was selected for further studies. In addition, the best primer and probe concentrations giving the lowest Ct value with an adequate fluorescence for a given target concentration were selected for further analyses. Therefore, F-idhtrb and R-idhtrb concentrations used for the SYBR Green reaction were 400 nM and

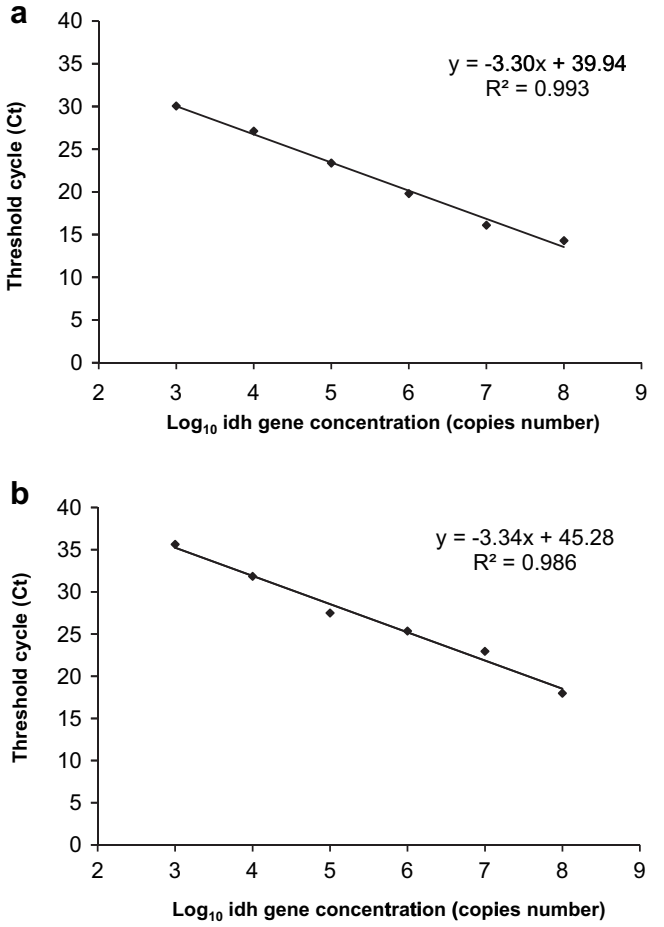

Fig. 1. Representative standard curves showing the correlation between the initial copy number of *idh* gene (obtained with dilutions of the 600-bp *idh* fragment) and threshold cycle (Ct) values of the SYBR Green (a) and TaqMan (b) Real-Time PCR methods for different concentration of *idh* gene copy. These curves were generated with three independent reactions.

Table 3
Efficiency of amplification and R^2 obtained from standard curves of conidia from patulin-producing molds in artificially inoculated foods by RTi-PCR.

Food sample	Patulin-producing species	RTi-PCR method	Efficiency (%)	Intercept (Ct)	R^2
Cooked ham	*P. expansum* CECT 2278	SYBR Green	125 ± 0.6[a]	36.0[b]	0.98
		TaqMan	112 ± 1.0	42.8	0.98
	A. flavus CECT 2684	SYBR Green	123 ± 1.7	35.9	0.98
		TaqMan	113 ± 1.3	40.0	0.97
	P. commune CECT 2264	SYBR Green	128 ± 3.7	35.6	0.99
		TaqMan	105 ± 2.7	42.5	0.98
Dry-fermented sausage "salchichón"	*P. expansum* CECT 2278	SYBR Green	112 ± 0.4	35.8	0.98
		TaqMan	84 ± 1.3	45.0	0.98
	A. flavus CECT 2684	SYBR Green	109 ± 1.4	35.9	0.98
		TaqMan	80 ± 0.8	44.7	0.98
Peach	*P. expansum* CECT 2278	SYBR Green	112 ± 1.7	39.2	0.98
		TaqMan	107 ± 2.9	43.3	0.97
	A. flavus CECT 2684	SYBR Green	113 ± 1.5	39.4	0.98
		TaqMan	110 ± 3.6	42.8	0.98

[a] Data represent the mean efficiency ± standard deviation of 3 independent assays each consisting of triplicate samples.
[b] Data represent the mean intercept of 3 independent assays each consisting of triplicate samples.

700 nM, respectively. For the TaqMan assays, different concentrations of primers and probe were also evaluated. The lowest Ct value was obtained with 450 nM of each primer and probe (data not shown).

3.3. Specificity of the RTi-PCR reactions using reference strains

The specificity of primers and probe was tested on genomic DNA from selected producing and non-producing species of *Aspergillus*, *Emericella*, *Penicillium* and *Rhizopus*. In the SYBR Green method, all patulin-producing strains showed Tm values ranging from 86.9 to 88.5 °C, mainly grouped close to these values, while those from non-producing strains ranged from 66.0 to 82.4 °C (Table 1). The specific PCR product was only obtained from patulin-producing molds.

Patulin producers showed Ct values lower than 31 and 36.5 for SYBR Green and TaqMan methods, respectively. In addition, for TaqMan assay, these values were significantly ($p < 0.05$) lower in patulin-producing strains than in non-producers.

3.4. Development of the RTi-PCR methods to quantify patulin producers

The conventional PCR method yielded a 600-bp PCR product (see Section 2.4) that was used as a standard to build the standard curve to calculate the number of copies of the *idh* gene produced in the SYBR Green and TaqMan assays.

Standard curves relating Ct values and the logarithm of template copies were generated for each optimized method. No significant differences ($p < 0.05$) were found between standard curves obtained by the different patulin producers since the slopes and R^2 were nearly identical (data not shown). For this, a standard curve with average values of slope and R^2 was used as representative for each method (Fig. 1). Good linear relationship between Ct values and the *idh* gene copy number was obtained for both RTi-PCR methods, with R^2 values close to 0.99 for SYBR Green and 0.98 for TaqMan assays. The slopes of the linear regression curves were −3.30 and −3.34 for SYBR Green and TaqMan assays, respectively (Fig. 1). The efficiency values were 100.9% for SYBR Green and 99.1% for TaqMan assays.

3.5. Sensitivity of the RTi-PCR assays on inoculated food matrices

The ability of the optimized RTi-PCR protocols to quantify patulin-producing molds was evaluated in artificially inoculated foods. Standard curves between Ct values and amount of DNA extracted from inoculated foods were constructed for each food matrix. The efficiency values for the SYBR Green assays ranged from 109 to 113%, except for cooked ham that reached values from 123 to 128%. Similarly, for TaqMan assays the efficiencies values for these foods ranged from 80 to 113% (Table 3). A good linear correlation was obtained over the range 1×10^4 to 10 conidia g^{-1} per reaction for RTi-PCR assays (Table 3). The detection limit was 10 conidia g^{-1} for both optimized RTi-PCR methods in all inoculated food matrices. After comparison of the fungal load, the number of *idh* copies obtained by RTi-PCR, and the number of inoculated spores in these food matrices, all differences were always lower than 1 log unit (data not shown).

The absence of PCR inhibitors was studied in inoculated foods by comparison of the amplification plots obtained from DNA extracted from a patulin producer with and without spiking with DNA extracted from a non-inoculated food sample. No significant differences ($p < 0.05$) were found between the Ct values obtained in these two tests (Table 4).

The ability of the designed RTi-PCR methods to quantify two levels of conidia inoculated on several foods is shown in Table 5. For most foods, the amount of conidia assessed by both RTi-PCR methods for the high inoculation level (3×10^3 conidia g^{-1}) was close to the inoculated amount. Only when the SYBR Green methodology was used in fruits, the number assessed exceeded up to 16% the inoculated amount. No effect was observed due to the different food matrices. At the high level of inoculation, the number

Table 4
Ct values for DNA from the patulin-producing *P. expansum* CECT 2278, with and without spiking with DNA from non-inoculated food samples.

RTi-PCR method	Fungal DNA + deionized water	Fungal DNA + DNA from cooked ham	Fungal DNA + DNA from "salchichón"	Fungal DNA + DNA from peach
SYBR Green	19.9 ± 0.12[a]	20.3 ± 0.63	19.9 ± 0.09	20.0 ± 0.41
TaqMan	20.5 ± 0.29	21.0 ± 0.38	20.7 ± 0.20	20.8 ± 0.26

No significant differences ($p < 0.05$) were found between Ct values obtained in the two different tests.
[a] Data represent the mean threshold cycle (Ct) ± standard deviation (SD) of 5 independent DNA extractions.

Table 5
Quantification of conidia from patulin-producing molds in artificially inoculated foods by RTi-PCR.

Inoculated conidia	RTi-PCR method	Food products							
		Cooked products[a]			Cured products[b]			Fruits[c]	
		Mortadella	Cooked turkey breast	Cooked ham	Dry-cured ham	"Salchichón" sausage	"Chorizo" sausage	Apple	Peach	Pear
3×10^3	SYBR Green	$3.3\ (0.32) \times 10^3$ [d]	$2.5\ (0.49) \times 10^3$	$2.7\ (0.33) \times 10^3$	$2.8\ (0.52) \times 10^3$	$2.8\ (0.49) \times 10^3$	$2.7\ (0.51) \times 10^3$	$3.3\ (0.62) \times 10^3$	$3.6\ (0.25) \times 10^3$	$4.5\ (0.63) \times 10^3$
	TaqMan	$3.0\ (0.58) \times 10^3$	$3.3\ (0.47) \times 10^3$	$3.1\ (0.68) \times 10^3$	$2.9\ (0.35) \times 10^3$	$3.2\ (0.42) \times 10^3$	$2.7\ (0.53) \times 10^3$	$3.1\ (0.72) \times 10^3$	$2.9\ (0.52) \times 10^3$	$2.7\ (0.66) \times 10^3$
3×10	SYBR Green	$3.6\ (0.42) \times 10$	$4.4\ (0.82) \times 10$	$4.1\ (0.64) \times 10$	$4.7\ (0.85) \times 10$	$4.4\ (0.53) \times 10$	$3.2\ (0.50) \times 10$	$3.9\ (0.56) \times 10$	$3.3\ (0.23) \times 10$	$3.8\ (0.28) \times 10$
	TaqMan	$3.8\ (0.91) \times 10$	$2.7\ (0.54) \times 10$	$2.5\ (0.64) \times 10$	$2.4\ (0.86) \times 10$	$3.3\ (0.35) \times 10$	$3.0\ (0.47) \times 10$	$3.8\ (0.75) \times 10$	$3.5\ (0.63) \times 10$	$3.7\ (0.72) \times 10$

Values in brackets represent standard deviation (±SD).
[a] Data obtained from standard curve for cooked ham inoculated with *P. commune* CBS 311.48.
[b] Data obtained from standard curve for dry-fermented sausage "salchichón" inoculated with *A. flavus* CECT 2684.
[c] Data obtained from standard curve for peach inoculated with *P. expansum* CECT 2278.
[d] Data represent the mean of 3 independent assays, each consisting of triplicate samples.

of spores estimated with the SYBR Green assay was significantly higher ($p < 0.05$) than that with the TaqMan protocol. At the low level of inoculation (3×10 conidia g^{-1}), data obtained were less disperse, including those obtained with SYBR Green in fruits. The standard deviation at the high level of inoculation were from 2.5×10^2 to 7.2×10^2 conidia g^{-1} and at the low level of inoculation were from 2.3 to 9.1 conidia g^{-1}. This indicates a good repeatability of the DNA extraction from artificially inoculated food.

3.6. Quantification of the fungal load and idh gene copies in inoculated foods

The ability of the designed RTi-PCR methods to quantify the fungal load in inoculated food samples was evaluated after different incubation times. The number of *idh* copies and the cfu values by plating were also determined. The results are shown in Fig. 2. The growth of *P. expansum* CECT 2278 in apple did not show a significant lag phase, reaching the stationary phase after only 7 days, whereas in dry-cured ham showed a 5-days lag phase. The natural fungal contamination of the samples was lower than 1 log cfu g^{-1}. The characterization of the isolates from non-inoculated foods revealed no typical colonies of *P. expansum*.

The results obtained showed a high correlation between the fungal count with both *idh* copies and the fungal load obtained by RTi-PCR. For SYBR Green assays, R^2 values between these three parameters were 0.972 and 0.975 in apple and dry-cured ham, respectively. For the TaqMan assays, R^2 values were 0.982 in apple and 0.964 in dry-cured ham. There was no significant difference between values of fungal count with those of the *idh* gene copies and the fungal load obtained by RTi-PCR. In addition, for both RTi-PCR methodologies, the standard deviation was always lower than 0.20 log (cfu or *idh* copies) for most of the incubation time, except for dry-cured ham at the first sampling time, where the standard deviation reached a value 0.38.

4. Discussion

To develop specific RTi-PCR assays for quantifying patulin-producing molds it is necessary to design specific primers. For this, the use of a gene implied in the patulin biosynthesis is necessary. In the present work, the *idh* gene was target for the development of specific RTi-PCR assays for detecting patulin-producing molds. This gene encodes the seventh enzyme involved in the patulin biosynthesis in *Penicillium* and *Aspergillus* spp. Furthermore, it has been demonstrated that the absence of this gene in other clusters related with the patulin cluster involves the non-production of this mycotoxin (Puel et al., 2010). Thus, the presence of the *isopoexidon dehydrogenase* seems to be necessary for patulin biosynthesis in both *Penicillium* and *Aspergillus* species.

From the two primer pairs designed in the present work, the most appropriate for RTi-PCR was F-idhtrb/R-idhtrb, since the Ct values obtained were lower than those with primer pair IDH1/R-idhtra for all assayed DNA quantities. With the former primer pair, most patulin-producing strains showed peak Tm around 88.0 °C, whereas the remaining patulin producers (18.9%) showed peak Tm of 86.9 and 87 °C. With this primer pair, Ct values showed also some differences within patulin-producing strains. However, Ct values found for producing strains were significantly ($P < 0.05$) lower than those for non-producing strains for TaqMan assay. Besides, in the SYBR Green assay, the Tm value differs between producers and non-producers. The divergences in Tm and Ct values can be due to small differences in the sequences of the target *idh* gene of the different species and genera analyzed. In relation to Tm, DNA melting curves are a function of the GC/AT ratio, length, and sequences (Ririe et al., 1997). In fact, the information available on

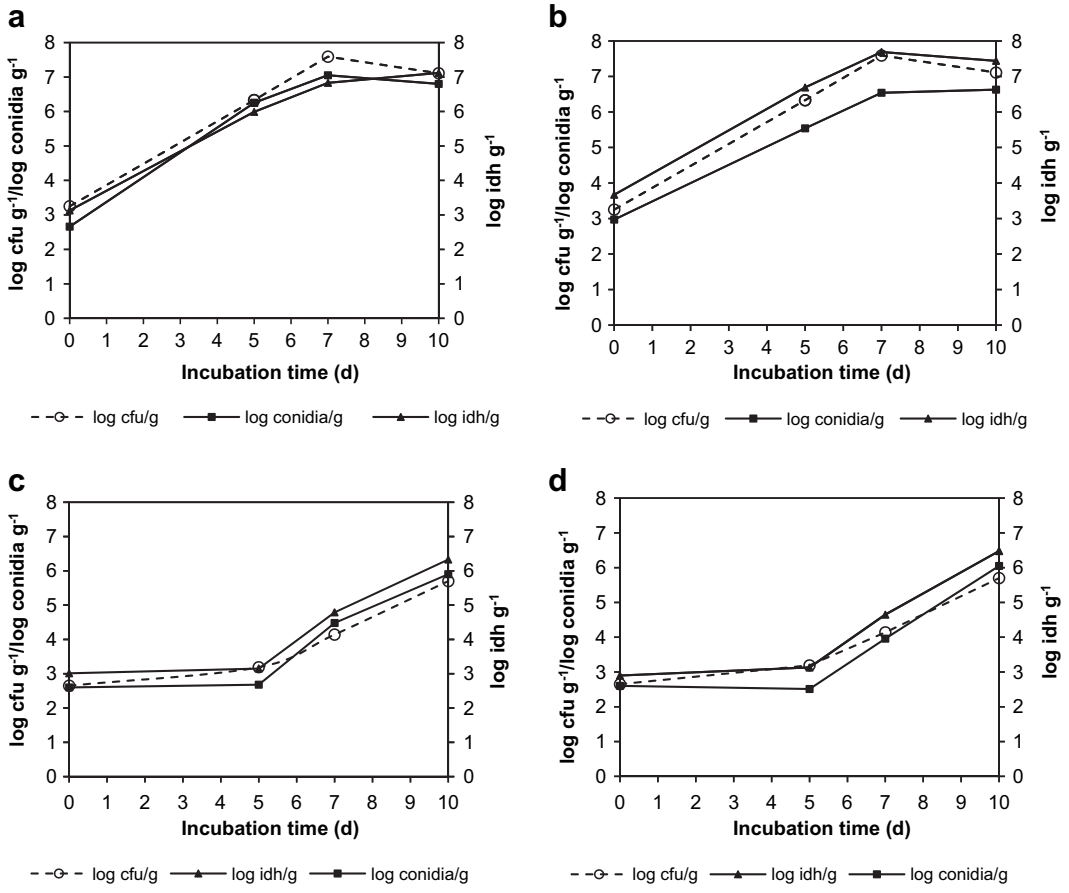

Fig. 2. Comparison of the fungal count (cfu data) with *idh* gene copy number and fungal load obtained by SYBR Green (2a, 2c) and TaqMan (2b, 2d) RTi-PCR of *P. expansum* CECT 2278 in inoculated apple (2a, 2b) and dry-cured ham (2c, 2d) during 10 days of incubation.

two of the strains used in the present work, *P. expansum* CECT 2280 and *P. expansum* CECT 2278, may support the differences in Tm values. According to the *idh* sequences for these two strains (Accession numbers AY885568 and AY885570), a difference of 1–2 °C could be expected. Even though the precise *idh* sequences for most of the reference strains used are not described, relevant differences have been reported for this gene within *Penicillium* spp. affecting more than 10 amino acids (Dombrink-Kurtzman, 2006, 2007). In relation to Ct values, the differences observed (Table 1) does not seem to affect the ability of the developed methods to quantify patulin-producing molds in foods, since their efficiency was nearly identical for two mold species (*P. expansum* CECT 2278 and *A. flavus* CECT 2684) that showed highly different Ct values with genomic DNA. This demonstrates that the developed methods based on the *idh* gene may be efficient to quantify the main patulin-producing strains in foods, in spite of the polymorphism found in the *idh* gene.

Results obtained with both RTi-PCR protocols were closely related to patulin detection by MECE and HPLC-MS. The detection limit for patulin detection with both MECE and HPLC-MS methods is 4 μg L^{-1}, that is enough for an accurate differentiation of producing and non-producing strains. Analyses carried out by MECE and HPLC-MS with the 62 strains from Culture Collections confirmed patulin production in all those identified as patulin producers by the culture collections. In addition, 22 strains with no information on patulin production from the culture collections produced this mycotoxin. This showed that patulin production is quite common in different food-related mold species, including *A. flavus*, *Aspergillus oryzae*, *A. tamarii*, *A. toxicarius*, *Penicillium aurantiogriseum*, *Penicillium camemberti*, *P. commune*, *P. expansum*, *Penicillium polonicum*, *Penicillium verrucosum*, *Emericella quadrilineata*, *E. rugulosa*, and *Emericella variecolor*. No amplification by RTi-PCR was observed for any of the non-patulin producers. The two optimized methods can be considered specific, providing a good discrimination between patulin-producing and non-producing strains across species and genera.

The functionality of the developed methods was demonstrated by the high linear relationship of the standard curves constructed with the *idh* gene copy number and Ct values for the different patulin producers tested. In addition, there was no significant difference in the slope and R^2 between standard curves generated with different molds, even though Ct values observed with genomic DNA showed several differences. This allows using either of the constructed standard curves for the different target strains for the quantification of *idh* copies. Although no guidelines have been established for standard curves used in RTi-PCR assays that measure fungi, Fredlund et al. (2008) and Suanthie et al. (2009) suggested the use of criteria established for Genetically Modified Organism analysis of foods where the slope of the standard curve should range between −3.1 and −3.6, corresponding to a PCR efficiency of 80 and 110% and the R^2 value be ≥0.98. These guidelines should also be valid for the analysis of fungal DNA in foods. In the present work, the two optimized methods had R^2 values around 0.98. Both SYBR Green and TaqMan assays showed slope values in the acceptable range and a good efficiency near to 100%, which indicates a good optimization of the PCR reaction and that no errors

occurred in this process. Similar values of efficiency have been reported for RTi-PCR protocols to detect mycotoxin-producing molds (Mulé et al., 2006; Fredlund et al., 2008).

The sensitivity of the RTi-PCR assays was tested in three different food matrices inoculated with 10-serial dilutions of known concentration of spores from three representative patulin-producing molds. The slopes of the standard curves and R^2 from all inoculated foods were within the recommended range suggested by Fredlund et al. (2008). The efficiency of the standard curves for SYBR Green assays in cooked ham ranged, however, from 123 to 128%. This fact could be due to the presence of PCR-inhibiting substances in this kind of foods, such as fat or carbohydrates (Rossmanith et al., 2007). In addition, the limit of detection in all inoculated foods was 10 conidia g^{-1} for both optimized RTi-PCR methods, which was very close to other previously reported values (Selma et al., 2008).

Thus, the results obtained in the present work show that both RTi-PCR methods could be applied for quantifying patulin-producing molds directly in foods without incubation time.

To test the ability of the RTi-PCR methods to quantify patulin-producing molds in foods, two known amounts of fungal conidia were inoculated. The accuracy obtained (Table 5) was in the same range as the RTi-PCR methods developed to detect toxigenic *Aspergillus carbonarius* (Selma et al., 2008). Given that conidia were inoculated directly on the food matrix instead of in a juice or food extract, both RTi-PCR methods developed were found satisfactory to cope with potential inhibitors present on food samples. In addition, low standard deviations revealed a good repeatability for both SYBR Green and TaqMan methods. Similar values have been reported for other RTi-PCR protocols developed to detect mycotoxin-producing molds when DNA was extracted from artificially inoculated or naturally contaminated samples (Nicolaisen et al., 2009; Gónzalez-Salgado et al., 2009).

Tannins, polysaccharides and pigments in fruits may influence quantification (Mulé et al., 2006) by interfering with PCR amplification (Fredlund et al., 2008; Demeke and Jenkins, 2010). However, no differences in the quantification of patulin-producing molds between the food matrices have been found in the present study.

The quantification of the fungal contamination in foods is a difficult task due to the nature of the fungal colony, which consists of filamentous mycelia cells and single celled spores. The assays to quantify the fungal load in apple and in dry-cured ham slices inoculated with target molds were designed to evaluate the efficiency of the developed methods in complex matrices, where just spores are not representative of fungal biomass due to hyphal development. Results of this assay showed that the calculated *idh* gene copy number and the fungal load obtained with the RTi-PCR methods were nearly identical to the fungal load determined by plating. Regarding accuracy of the developed methods when applied to foods, the mean values of fungal load, including conidia and mycelium, quantified with SYBR Green and TaqMan methodologies were close to the inoculated amount. Similar results have been reported for other RTi-PCR protocol developed to quantify the copy number of *nor-1* gene of *A. flavus* in foods (Mayer et al., 2003).

Both SYBR Green and TaqMan RTi-PCR procedures developed in the present study could be carried out in a relatively short time period (5–6 h for DNA extraction and 2–3 h for RTi-PCR). In this way, both methods allow a reliable rapid estimation of the contamination with patulin-producing molds for evaluating the quality of raw materials/ingredients, such as apple juice or apple pulp, and monitoring of hygienic control on mold-ripened foods. Thus, the methods designed would serve to determine the contamination by patulin producers even before fungal development can be observed, which would be very useful for preventing toxin production during fruit storage and dry-ripening or fermenting in foods. Patulin production has been reported at temperatures from 4 to 25 °C in foods, reaching unacceptable levels in just two days, with no evident fungal growth (Baert et al., 2007; Morales et al., 2007). Such temperatures are very usual throughout fruit storage and ripening processes. Thus, even a low contamination with patulin-producing molds could become a serious problem in these kinds of foods. The sensitivity of the developed methods can be sufficient for monitoring the target molds in HACCP programs. The ability to efficiently quantify patulin-producing strains at levels as low as 30 spores g^{-1}, even in foods heavily contaminated with non-target molds, can be adequate for the food industry to take the appropriate corrective actions, prevent fungal growth, and minimize the hazard due to patulin formation. Thus, quantification of a low level of patulin producer molds allow to classify ingredients for an urgent processing or to be stored as required. Since there is no current industrial process to detoxify foods contaminated with patulin, ensuring the adequate level of control on the producing molds can be regarded as essential, particularly for apple-derived ingredients, where the toxigenic potential of fungal contaminants is difficult to assess.

In conclusion, both RTi-PCR procedures developed in this study could be used for specific quantification of patulin-producing molds in foods due to its high specificity and sensitivity that can be applied to various food matrices.

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References

Atoui, A., Mathieu, F., Lebrihi, A., 2007. Targeting a polyketide synthase gene for *Aspergillus carbonarius* quantification and ochratoxin A assessment in grapes using real-time PCR. Int. J. Food Microbiol. 115, 313–318.

Baert, K., Devlieghere, F., Flyps, H., Oosterlinck, M., Ahmed, M.M., Rajković, A., Verlinden, B., Nicolaï, B., Debevere, J., De Meulenaer, B., 2007. Influence of storage conditions of apples on growth and patulin production by *Penicillium expansum*. Int. J. Food Microbiol. 119, 170–181.

Demeke, T., Jenkins, R., 2010. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. Anal. Bioanal. Chem. 396, 1977–1990.

Dombrink-Kurtzman, M.A., 2006. The isoepoxydon dehydrogenase gene of the patulin metabolic pathway differs for *Penicillium griseofulvum* and *Penicillium expansum*. Anton. Leeuw. Int. J. G. 89, 1–8.

Dombrink-Kurtzman, M.A., 2007. The sequence of the isoepoxydon dehydrogenase gene of the patulin biosynthetic pathway in *Penicillium* species. Anton. Leeuw. Int. J. G. 91, 179–189.

Erdogan, A., Sert, S., 2004. Mycotoxin-forming ability of two *Penicillium roqueforti* strains in blue moldy tulum cheese ripened at various temperatures. J. Food Protect 67, 533–535.

Erdogan, A., Gurses, M., Sert, S., 2003. Isolation of moulds capable of producing mycotoxins from blue mouldy Tulum cheeses produced in Turkey. Int. J. Food Microbiol. 85, 83–85.

Fredlund, E., Gidlund, A., Olsen, M., Börjesson, T., Spliid, N.H.H., Simonsson, M., 2008. Method evaluation of *Fusarium* DNA extraction from mycelia and wheat for down-stream real-time PCR quantification and correlation to mycotoxin levels. J. Microbiol. Meth. 73, 33–40.

Gil-Serna, J., Gónzalez-Salgado, A., Gónzalez-Jaén, M.T., Vázquez, C., Patiño, B., 2009. ITS-based detection and quantification of *Aspergillus ochraceus* and *Aspergillus westerdijkiae* in grapes and green coffee beans by real-time quantitative PCR. Int. J. Food Microbiol. 131, 162–167.

Gónzalez-Salgado, A., Patiño, B., Gil-Serna, J., Vázquez, C., Gónzalez-Jaén, M.T., 2009. Specific detection of *Aspergillus carbonarius* by SYBR Green and TaqMan quantitative PCR assays based on the multicopy ITS2 region of the rRNA gene. FEMS. Microbiol. Lett. 295, 57–66.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Stahlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. Mol. Aspects. Med. 27, 95–125.

Kure, C.F., Skaar, I., Brendehaug, J., 2004. Mould contamination in production of semi-hard cheese. Int. J. Food Microbiol. 93, 41–49.
Labuda, R., Tančinová, D., 2006. Fungi recovered from slovakian poultry feed mixtures and their toxinogenity. Ann. Agr. Env. Med. 13, 193–200.
Martín, A., Jurado, M., Rodríguez, M., Núñez, F., Córdoba, J.J., 2004. Characterization of molds from dry-cured meat products and their metabolites by micellar electrokinetic capillary electrophoresis and random amplified polymorphic DNA PCR. J. Food Protect 67, 2234–2239.
Mayer, Z., Bagnara, A., Färber, P., Geisen, R., 2003. Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. Int. J. Food Microbiol. 82, 143–151.
Morales, H., Marín, S., Centelles, X., Ramos, A.J., Sanchis, V., 2007. Cold and ambient deck storage prior to processing as a critical point for patulin accumulation. Int. J. Food Microbiol. 116, 260–265.
Mulé, G., Susca, A., Logrieco, A., Stea, G., Visconti, A., 2006. Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. Int. J. Food Microbiol. 111, S28–S34.
Nicolaisen, M., Supronienė, S., Nielsen, L.K., Lazzaro, I., Spliid, N.H., Justesen, A.F., 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. J. Microbiol. Meth. 76, 234–240.
Niessen, L., 2007. PCR-based diagnosis and quantification of mycotoxin producing fungi. Int. J. Food Microbiol. 119, 38–46.
Núñez, F., Rodríguez, M.M., Bermúdez, E., Córdoba, J.J., Asensio, M.A., 1996. Composition and toxigenic potential of the mould population on dry-cured Iberian ham. Int. J. Food Microbiol. 32, 185–197.
Paterson, R.R.M., 2004. The isoepoxydon dehydrogenase gene of patulin biosynthesis in cultures and secondary metabolites as candidate PCR inhibitors. Mycol. Res. 108, 1431–1437.
Paterson, R.R.M., Archer, S., Kozakiewicz, Z., Lea, A., Locke, T., O'Grady, E., 2000. A gene probe for the patulin metabolic pathway with potencial use in novel disease control. Biocontrol. Sci. Techn 10, 509–512.
Powell, H.A., Gooding, C.M., Garret, S.D., Lund, B.M., McKee, R.A., 1994. Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. Lett. Appl. Microbiol. 18, 59–61.
Puel, O., Galtier, P., Oswald, I.P., 2010. Biosynthesis and toxicological effects of patulin. Toxins 2, 613–631.
Puel, O., Tadrist, S., Delaforge, M., Oswald, I.P., Lebrihi, A., 2007. The inability of *Byssochlamys fulva* to produce patulin is related to absence of 6-methylsalicylic acid synthase and isoepoxydon dehydrogenase genes. Int. J. Food Microbiol. 115, 131–139.
Ririe, K.M., Rasmussen, R.P., Wittwer, C.T., 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reactions. Anal. Biochem. 245, 154–160.
Rossmanith, P., Süβ, B., Wagner, M., Hein, I., 2007. Development of matrix lysis for concentration of gram positive bacteria from food and blood. J. Microbiol. Methods 69, 504–511.
Sánchez, B., Rodríguez, M., Casado, E.M., Martín, A., Córdoba, J.J., 2008. Development of an efficient fungal DNA extraction method to be used in random amplified polymorphic DNA-PCR analysis to differentiate cyclopiazonic acid mold producers. J. Food Protect 71, 2497–2503.
Selma, M.V., Martínez-Culebras, P.V., Aznar, R., 2008. Real-time PCR based procedures for detection and quantification of *Aspergillus carbonarius* in wine grapes. Int. J. Food Microbiol. 122, 126–134.
Selmanoglu, G., 2006. Evaluation of the reproductive toxicity of patulin in growing male rats. Food Chem. Toxicol. 44, 2019–2024.
Selmanoglu, G., Koçkaya, E.A., 2004. Investigation of the effects of patulin on thyroid and testis, and hormone levels in growing male rats. Food Chem. Toxicol. 42, 721–727.
Sosa, M.J., Córdoba, J.J., Díaz, C., Rodríguez, M., Bérmudez, E., Asensio, M.A., Núñez, F., 2002. Production of cyclopiazonic acid by *Penicillium commune* isolated from dry-cured ham on a meat extract-based substrate. J. Food Protect 65, 988–992.
Suanthie, Y., Cousin, M.A., Woloshuk, C.P., 2009. Multiplex real-time PCR for detection and quantification of mycotoxigenic *Aspergillus*, *Penicillium* and *Fusarium*. J. Stored. Prod. Res. 45, 139–145.
Sweeney, M.J., Dobson, A.D.W., 1998. Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. Int. J. Food Microbiol. 43, 141–158.
Tsao, R., Zhou, T., 2000. Micellar electrokinetic capillary electrophoresis for rapid analysis of patulin in apple cider. J. Agric. Food Chem. 48, 5231–5235.