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Polyphasic identification of *Penicillium* spp. isolated from Spanish semi-hard ripened cheeses

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ABSTRACT

Fifteen samples of semi-hard ripened cheeses, both spoiled (10) and unspoiled (5), and obtained from cheese factories located in Northwest of Spain, were analysed by a dilution plating technique and direct sampling. A total of 32 isolates were identified at species level by a polyphasic approach (phenotypic characterization, partial extrolite analysis and molecular identification). Most isolates (65.6%) belonged to the species *P. commune*; other species found were *P. solitum*, *P. chrysogenum*, *P. nordicum*, *P. expansum* and *P. cvjetkovicii*. All of the *P. commune* isolates were able to produce cyclopiazonic acid, while the *P. nordicum* and the *P. expansum* isolates were producers of ochratoxin A and patulin respectively. Despite this, the role of *P. commune* as beneficial fungi in cheese ripening should be investigated. Molecular identification based on *BenA* sequence analysis was able to identify the majority of isolates. The three mycotoxins investigated can be considered key for identification. The polyphasic approach seems to be a very valuable tool for identification of isolates of this complex genus.

1. Introduction

Cheese is an excellent substrate for mould growth. Aside from mould's role in the production of some cheese varieties (i.e., *Penicillium roqueforti* in blue cheeses and *P. camemberti* in white coat cheese), some mould species are responsible for spoilage and impact overall product quality (Bullerman, 1981; Stark, 2007). This dairy product can deteriorate due to the presence of visible colonies on the surface or in small fissures close to it, producing off-flavours. This loss of quality has economic consequences in addition to its sensory effects. Moulds may originate from raw materials such as milk or may be introduced, mainly from the environment, during cheese making (Hymery et al., 2014). Mould growth can be observed on cheese during ripening (this is quite common in some Spanish cheese factories), storage and distribution for retail, and even at the consumer level (Bullerman, 1981). In addition, the growth of fungi may also represent a health risk for the consumer, since many species are able to produce mycotoxins (Bullerman, 1981; Lund et al., 1995).

Penicillium is considered to be the most frequent fungal genus to contaminate cheese (Bullerman, 1981; Frisvad et al., 2007a; Frisvad and Samson, 2004; Lund et al., 1995; Pitt and Hocking, 2009). Identification of members of this genus at species level is a very complex task, as most species have very similar properties. Conventional identification is based on morphological studies (both macroscopic and microscopic) and phenotypic characterization (growth at different temperatures, presence of pigment and exudate, and cultivation methods) (Pitt, 1979). Recently, molecular identification has arisen to assist with identification. It offers the advantage of measuring stable genotypic characteristics and being independent of culture conditions and operator interpretation (Perrone and Susca, 2017). Frisvad and Samson (2004) proposed a polyphasic approach to identify species of *Penicillium* subgenus *Penicillium* (which includes most species that cause cheese spoilage) based on morphological, chemical and molecular analysis.

According to Frisvad and Samson (2004), Frisvad et al. (2007a) and Pitt and Hocking (2009), there are 15 species of *Penicillium* subgenus *Penicillium* associated with cheese spoilage; these belong to the five sections reviewed by Houbraken et al. (2016) (Table 1). They grow in colonies on Malt Extract Agar (MEA)that are some shade of green when they, with branched conidiophores (*ter-* to *quater-verticillata*) and flask-shaped phialides (subgenus *Penicillium*). Most of these species are mycotoxigenic (Table 1), with some producers of mycotoxins found in

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Table 1
Species of *Penicillium* (subgenus *Penicillium*) associated to cheese spoilage and mycotoxins associated to this food (according to Frisvad and Samson, 2004; Frisvad et al., 2007a, b; Pitt and Hocking, 2009; Houbraken et al., 2016).

Section *Brevicompacta*	Section *Roquefortorum*	Section *Chrysogena*	Section *Penicillium*	Section *Fasciculata*
Series *Olsonii* *P. brevicompactum*[a]	Series *Roqueforti* *P. roqueforti*[abch]	Series *Chrysogena* ***P. chrysogenum***[b] *P. nalgiovense*	Series *Expansa* ***P. expansum***[bdi]	Series *Viridicata* *P. viridicatum*[e] Series *Verrucosa* ***P. nordicum***[f] *P. verrucosum*[f i] Series *Camemberti* ***P. solitum*** *P. discolor* *P. echinulatum* ***P. commune***[g] *P. palitans*[cg] *P. crustosum*[b] *P. atramentosum*[b]

[a] Mycophenolic acid.
[b] Roquefortine C.
[c] Isofumigaclavine.
[d] Patulin.
[e] Penicillic acid.
[f] Ochratoxin A.
[g] Cyclopiazonic acid.
[h] PR toxin.
[i] Citrinin. In **bold**, species found in this study.

Table 2
Origin of the *Penicillium* isolates identified.

Cheese Sample n = 15	Type	S/NS	Isolates n = 32	Identification n = 32
Q1, Q4, Q8, Q9, Q10, Q11, Q12, Q13, Q14, Q15	Semi-hard, ripened without fungal culture added (cylindrical, “Castellano” type).	S	P1, P2, P3, QLM1, QLM2, M35, M170, M30, M34, M32, M76, M123, M124, M57, M145, Q3M1 (n = 16)	*P. commune* (9) *P. solitum* (3) *P. chrysogenum* (2) *P. nordicum* (1) *P. cvjetkovicii* (1)
Q3, Q5, Q6, Q7	Semi-hard, ripened, blue coat without surface fungal culture added (mostly, rectangular, “pata de mulo”)	US	Q2M1, Q2M2, Q2M3, Q2M4, Q2M5, Q2M11, QP1, QP2, QP3, QPA3, QPA4, QZ1, Q2M7 (n = 13)	*P. commune* (9) *P. solitum* (3) *P. expansum* (1)
Q2	Soft cheese, with blue coat without surface fungal culture added	US	P4, P5, P6 (n = 3)	*P. commune* (3)

S, spoiled; US, unspoiled.

cheese by different authors (reviewed by Hymery et al. (2014) and Weidenbörner (2008) (Table 2). Very few species belonging to other subgenera are associated with cheese. This is the case of *P. glabrum*, or the recently described *P. cvjetkovicii*, both of which belong to the subgenera *Aspergilloides*, *monoverticillata* (Peterson et al., 2015; Pitt and Hocking, 2009) and *P. citrinum,* subgenus *Furcatum* (Decontardi et al., 2017; Sinha and Ranjan, 1991).

Some popular varieties of cheese (semi-hard cheeses such as Castellano) made in the Northwest of Spain with raw ewe's milk have a variable ripening period, which can last several months under environmental conditions that allow the growth of moulds on the surface. This creates a blue-greyish coat that is periodically removed with a brush. In some similar varieties, the mould remains in the final product.

There are no previous studies on the fungal microbiota of Castellano cheese (the main variety included in our study). Thus, the purpose of the present study was to identify the fungi isolated from both spoiled and unspoiled ripened cheeses using polyphasic identification, with a view to contributing to the knowledge of these contaminants, their potential toxigenicity and the usefulness of the current identification techniques.

2. Materials and methods

2.1. Cheese samples and isolation of fungi

Fifteen samples of cheese (ten showing signs of spoilage, mostly blue, and five without spoilage but natural superficial mould growth, i.e., without the addition of any fungal culture) were obtained from seven different factories located in the Northwest of Spain (provinces of León and Zamora) during or at the end of the production process (3 spoiled samples came from consumers) (Table 2). Most samples were of pressed semi-hard cheeses made with raw ewe's milk, and one sample was a soft cheese (Table 2). Isolation of the strains was carried out by different techniques: dilution plating (after homogenization of 10 g of cheese in 90 mL of 0.1% peptone water solution and further 10-fold dilution), direct plating that involved the transfer of small cheese particles to agar plates, and use of adhesive tape to take a sample from the surface and place it on agar plates (Samson et al., 2010). Malt Extract Agar (MEA, Oxoid Thermo Fisher, UK) and Glucose Chloramphenicol Agar (GCA, Scharlab, Spain) were used as plating media. After incubation (25 °C/5-7 d), up to three colonies with different morphology per sample were selected and inoculated on MEA plates until pure cultures were obtained. Isolates were kept at 4 °C on MEA slants until identification.

2.2. Identification of fungi

Identification at genus level of selected isolates was done according to Samson et al. (2010) and to the macro- and microscopic characteristics. Identification at species level was carried out using a polyphasic approach (Frisvad and Samson, 2004; Visagie et al., 2014) consisting in a morphological characterization according to the keys and descriptions of Frisvad and Samson (2004), Frisvad et al. (2007a), Pitt and Hocking (2009), and Westerdijkinstitute (2018), as well as in extrolite analysis (CPA, OTA and PAT, selected according to their relevance to the species associated with cheese) (Table 1) and DNA barcoding.

2.2.1. Morphological characterization

Isolates were three-point inoculated onto the following media: Czapek Yeast Autolysate (CYA) agar, Yeast Extract Sucrose (YES) agar, MEA and Creatine Sucrose (CREA) agar (Frisvad and Samson, 2004). The plates were incubated for 7 d at 25 °C and also at 30 °C (CYA plates). After incubation, the following macromorphological characters were studied: colony diameter, texture, colour of conidia, obverse and reverse colours, soluble pigment, degree of growth and acid/base production on CREA.

Microscope slides were prepared from MEA cultures using lactic acid (60%) as mounting fluid, and the following micromorphological characters were studied: degree of branching of the conidiophores; dimension, shape and texture of stipes; and ornamentation of stipes and conidia.

2.2.2. OTA, CPA and PAT analysis

All the strains were assessed for production of three mycotoxins (OTA, CPA and PAT). These are 3 of the 9 mycotoxins that can be found in cheese according to the literature (Frisvad and Samson, 2004; Hymery et al., 2014) and are particularly useful for differentiating species belonging to section *Fasciculata* (Table 1), and, in particular, *P. commune*. Detection by thin layer chromatography was carried out after incubation of the isolates on YES plates for 7–14 days using the agar plug technique described by Samson et al. (2010). Aluminium plates (silica gel 60 F254; Merck, Germany) were directly used for OTA and PAT analysis. For CPA detection the plates were previously submerged into 10% oxalic acid in methanol for 2 min and heated in an oven at 110 °C/2 min (Gqaleni et al., 1996). Inoculation of the plates was carried out using a Camag Nanomat 4 (Camag, Switzerland).The standards for CPA and PAT were obtained from Sigma (Sigma-Aldrich Merck, Spain) and for OTA from Cayman Chemical (Cayman Chemical Company, USA). In the case of a negative result, the whole content of an agar plate was extracted with 50 mL of dichloromethane/methanol (80:20 v/v), homogenized, and filtered, and the solvent was evaporated under vacuum to dryness (Gqaleni et al., 1996). The extract was dissolved in 1 mL methanol and 10 μL were inoculated on the TLC plates. The mobile phase was TEF (toluene/ethyl acetate/90% formic acid, 5:4:1) (Samson et al., 2010). After drying, plates were treated as follows: OTA (NH_3 vapours for 2 min; fluorescent blue-turquoise spots were observed under ultraviolet light) (Frisvad et al., 1989); CPA (pulverization with Ehrlich reagent; a violet-blue spot was observed after some minutes) (Gqaleni et al., 1996); PAT (pulverization with 0.5% 3-methyl-2-benzothiazolinone hydrazone (MBTH) and heating in an oven at 105 °C/10 min; a yellow spot appears in visible light) (Frisvad et al., 1989). The detection limit was 10 μg/ml.

2.2.3. Ehrlich test

The Ehrlich test was conducted on all the strains via the filter paper method described by Lund (1995). Ehrlich reagent was prepared with 4-dimethylaminobenzaldehyde (Sigma-Aldrich Merck, Spain), dissolved in 96% ethanol and 37% hydrochloric acid (both from Panreac Química, Spain). After 2-10 min, a violet ring appears in case of a positive result. Some fungi produce alkaloids that will react with the Ehrlich reagent to give pink to red or yellow rings (Frisvad and Samson, 2004).

2.2.4. Molecular identification by DNA barcoding

Penicillium isolates were cultured on slants of MEA at 25 °C for 7 days. The mycelium was collected with 5 mL of sterile 0.05% Tween 80. Then, 2 mL were transferred to an Eppendorf vial and centrifuged at 16000 g/3 min. The pellet was washed twice with 1 mL bidistilled water, suspended in 250 μL of Instagene matrix (Bio-Rad, USA), and the DNA was extracted by heating for 3 h at 56 °C and 10 min at 95 °C. After vortex mixing and centrifugation at 12000 g/3 min, the supernatant was transferred to a fresh tube and 5 μL were used for PCR amplification (Ciardo et al., 2007). PCR was performed in 25 μL reactions in a Mastercycler Personal (Eppendorf Iberica, Spain). Amplification of ITS region, β-tubulin *BenA* gene, and calmodulin *CaM* gene was performed using the primers and conditions described by Visagie et al. (2014). PCR products were purified by NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). Both strands were sequenced in a MegaBACE 500 sequencer (GE Healthcare Life Sciences, UK). Strain identification was done by BLAST search against the RefSeq database for ITS sequences and a verified database for β-tubulin *BenA* and calmodulin *CaM* sequences (Visagie et al., 2014).

Phylogenetic trees were constructed using the UPGMA method, with the distances estimated by the Kimura 2-parameter model and a bootstrapping of 1000 replications using MEGA7 software (Kumar et al., 2016).

A collection strain of *P. verrucosum* CECT 20766 was included in the study, to help distinguish this species from *P. nordicum*.

3. Results and discussion

A total of 32 isolates were obtained and identified as belonging to the genus *Penicillium*, 16 of which were isolated from cheeses showing signs of spoilage and 16 of which were from non-spoiled cheeses (Table 2). Identification at the species level was achieved by morphological characterization and analysis of OTA, CPA and PAT, and DNA barcoding. Table 3 shows the results of the phenotypic and extrolite analysis of the isolates compared to those of Frisvad and Samson (2004); Supplementary Table 1 shows the results of the polyphasic identification, and Fig. 1 and Supplementary Fig. 1 show the results of the phylogenetic analysis based on the sequences of the *BenA* gene and ITS, respectively.

The species found in our study were *P. commune* (21 isolates, 65.6%), *P. solitum* (6 isolates, 18.8%), *P. chrysogenum* (2 isolates, 6.3%), *P. nordicum* (1 isolate, 3.1%), *P. expansum* (1 isolate, 3.1%), and *P. cvjetkovicii* (1 isolate, 3.1%) (Table 2).

Twenty-one isolates were identified as *P. commune* after polyphasic identification, nine obtained from spoiled cheeses and twelve from unspoiled cheeses (Table 2). They were all CPA producers, which is in accordance to the description of the species (Table 3) (this is one of the diagnostic features useful to differentiate *Penicillium* species related to cheese spoilage.). Five species of *Penicillium* are producers of CPA (Frisvad and Samson, 2004), but only *P. commune* and *P. palitans* are of importance as contaminants of cheese (Table 1). *P. camemberti* also produces CPA, but it is considered a non-contaminant. This species is used in the manufacture of soft cheeses and is rarely found outside the local environment of the manufacture of such varieties (Pitt and Hocking, 2009). It is not considered a spoilage agent. Furthermore, one of the diagnostic features of *P. camemberti* is the white or more rarely white-green floccose colour of the colonies on CYA (our isolates were all blue to blue-green; see Table 3). All of our *P. commune* strains produced a violet reaction in the Ehrlich test and a moderate to good acid production on creatine; one exhibited the reaction only under the colony, an exception contemplated by Frisvad and Samson (2004). Microscopically they showed rough-walled stipes and globose to subglobose conidia (Table 3). All of this complies with the characteristics of two species (*P. commune* and *P. palitans*). *P. palitans* could be

Table 3
Main phenotypic and selected extrolite characteristics of the *Penicillium* subgenus *Penicillium* isolated from cheese compared to the description of the species by Frisvad and Samson (2004).

Species	Origin[a]	CYA (mm)[b]	CYA (mm)[c]	YES (mm)[b]	MEA (mm) [b]	CREA (mm) [b] Growth/ Acid-base production	CPA/ OTA/PAT on YES[b]	Ehrlich reaction[b]	Conidium Color CYA [b]	Reverse color CYA [b]	Reverse color YES [b]	Stipes	Conidia
P. commune	A n = 21	9–39	0–5.5 (10)	25–40	15–26	9–12.7 Good/Acid	CPA	Violet, mostly strong	Blue green to green	Cream to cream yellow	Cream/ yellow/light brown[d]	Rough	Subglobose, smooth
	B	(15-) 21-35	0–4	29–50	(16-) 20-37	14-28 Very good/Strong acid	CPA	Strong violet	Blue green to green	Cream to beige or cream-yellow	Cream to yellow	Rough	subglobose to ellipsoidal smooth
P. chrysogenum	A n = 2	37–40	25–27	40–50	30–35	10–27	-	yellow	Blue green to green	Pale yellow/yellow	Pale yellow	Smooth	Smooth globose to subglobose
	B	23–46	14–27	40–64	19–52	16-26 Weak/None or poor acid, no base	-	None or yellow	Blue green to green	Cream, yellow, rarely brown	Citrine yellow	Smooth	Globose to subglobose to broadly ellipsoidal smooth
P. expansum	A n = 1	40	0	50	40	24 Good/Good acid followed by base production	PAT	Violet	Blue green to green	Orange brown	Cream yellow	Smooth	Smooth, ellipsoidal
	B	26–50	0–3	38–65	16–34	23-28 Very good (poor in few strains)/Good acid followed by base production	PAT	Strong violet	Blue green to green	Cream to yellow with brown center, orange brown or dark brown	Cream yellow or orange	Smooth (occasionally, rough)	Smooth, ellipsoidal
P. nordicum	A n = 1	11–13	0	15–20	12–13	12-13 None	OTA	Weak yellow	Green	Cream to light brown	Cream yellow	Rough	Smooth, globose to subglobose
	B	8–21	0	14–36	6–16	6-12 Weak/None	OTA	Yellow green	Green	Cream often with brown center	Cream yellow	Rough	Smooth-walled, globose to subglobose
P. solitum	A n = 6	11–28	0	24–38	13–25	8–15 Good to very good/Good acid	-	None	Green to blue green	Pale/pale to orange	Yellow to orange[e]	Rough	Smooth
	B	16–34	0	25–39	14–26	6–22 Good to very good/Under colony or good, base production poor or delayed	-	None	Dark blue green to green, cream-yellow exudates often	Cream to light beige	Yellow to orange	Rough	Smooth to slightly rough

[a] A, cheese isolates; B, reference data.
[b] , incubation at 25 °C for 7 d.
[c] , incubation at 30 °C for 7 d.
[d] , three isolates showed light brown color.
[e] , one isolate yellow with brown centre.

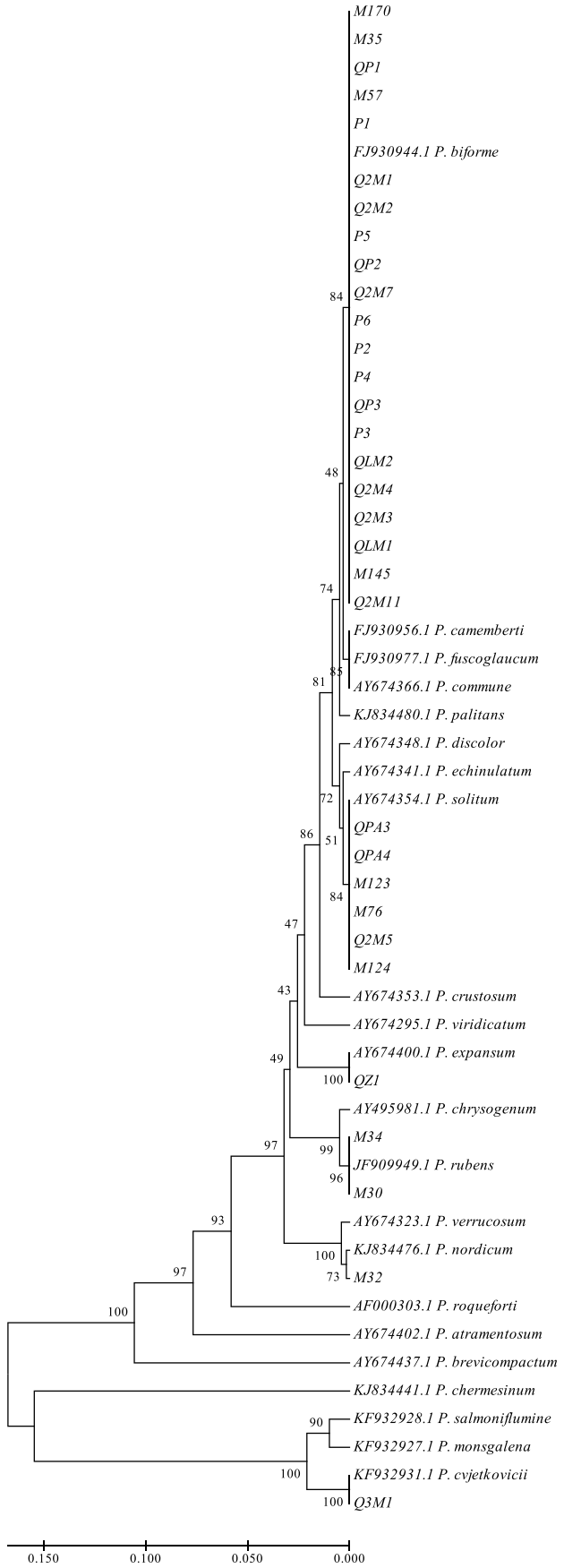

Fig. 1. UPGMA tree obtained from the phylogenetic analysis of the *BenA* sequences. Verified sequences from species of *Penicillium* associated with cheese spoilage were included. Bootstrapping values are shown in branch nodes.

differentiated by the brown centre in the reverse of the CYA plates, a feature that was not found in our isolates (nevertheless, this feature does not seem to be very consistent). Regarding identification by DNA barcoding, ITS sequencing was of limited use, as it was unable to differentiate *P. commune* from other species of *Penicillium* (Supplementary Fig. 1); however, *BenA* phylogenetic analysis allowed identification of all isolates, with a total agreement with the phenotypic approach (Supplementary Table 1 and Fig. 1). Differentiation between *P. commune* and *P. camemberti* and synonymised species such as *P. fuscoglaucum* or *P. biforme* was not possible with the molecular analysis, even using a third gene marker (*CaM*; Supplementary Table 1), and final identification was done according to phenotypic characteristics. The description of other genetic markers such as the microsatellite PC4 loci would be of help in the recognition of these closely related species (Giraud et al., 2010).

The primary habitat for *P. commune* in foods is cheese, and it is a major cause of spoilage (Filtenborg et al., 1996; Frisvad and Samson, 2004; Pitt and Hocking, 2009). Lund et al. (1995) found it dominant in an extensive study of different European cheeses (42% out of 371 isolates) and regarded it a spoiler. Other authors found *P. commune* as a predominant spoiler, for example, Tzanetakis et al. (1987) in a traditional Greek cheese, Kure (2001) and Kure et al. (2004) in semi-hard cheese, Hayaloglu and Kirbag (2007) in Turkish Kuflu cheese and Panelli et al. (2012) in Taleggio cheese. In contrast, Decontardi et al. (2018) found *P. commune* only in 7% of samples of crusts of Italian grana cheese, with *P. solitum* being dominant (55%). Identification was based on the calmodulin *CaM* gene, which can be useful for identification of isolates of *P. viridicatum* but is unable to differentiate isolates of the section *Viridicata* such as *P. solitum* or *P. commune*, as was concluded by Prencipe et al. (2018). In addition, no extrolite analysis was performed, which could have been useful in the identification. *P. commune* strains were isolated from different sources in cheese factories (equipment, plastic film and, principally, air) by Kure et al. (2002).

Some authors consider this species a part of the essential microflora of cheese that possibly contributes to the ripening changes and flavour characteristics of the final product (e.g. Kopanisti cheese and Taleggio cheese) (Hymery et al., 2014; Panelli et al., 2012; Tzanetakis et al., 1987). In the case of the samples of unspoiled cheese analysed in our study, they show a blue coat on the surface that could be due mainly to the presence of *P. commune,* according to our results (the cheeses had not been inoculated artificially and the mould that developed was part of a natural contamination). These cheeses are consumed and enjoyed by consumers, and therefore this fungus seemingly does not influence the organoleptic characteristics (flavour) of these varieties in a negative way. The prevalence of *P. commune* in cheeses is explained by its ability to grow at low temperatures, its low oxygen concentrations, its lipolytic activity and its resistance to the action of preservatives (Pitt and Hocking, 2009).

P. commune is a mycotoxigenic species. The production of CPA is considered a definite trait, though probably one of minor risk for consumers (Pitt and Hocking, 2009). In an extensive survey carried out on isolates obtained from cheese factories, it was found that 94% of *P. commune* isolates were CPA producers (Lund et al., 2003). CPA is a potent mycotoxin that in high concentrations produces focal necrosis in most vertebrate inner organs (Frisvad et al., 2007b; Perrone and Susca, 2017). It was also proposed that CPA was responsible for the severe effects on the muscles and bones of turkeys affected by the Turkey X disease, which was associated with peanuts that had been contaminated with aflatoxins (Jand et al., 2005). The target organs are kidneys and the gut tract in mammals; in humans, CPA is suspected to be responsible for acute mycotoxicosis (named “kodua”) that induces nerve troubles (Hymery et al., 2014). This mycotoxin is considered stable in cheese (Sengun et al., 2008). CPA has been found in Camembert and Brie cheese (Ansari and Häubl, 2016; Le Bars, 1979; Schoch et al., 1983), which is not unexpected, since *P. camemberti,* a domesticated species derived from *P. commune*, is a producer (Pitt et al., 1986), and it has

also been found in other varieties, such as Kasar cheese (Aran and Eke, 1987) and Taleggio cheese (Finoli et al., 1999). CPA is not under regulation in the European Union (European Commission, 2006).

Six isolates were identified as *P. solitum* (both from spoiled cheeses, three isolates, and unspoiled cheeses, three isolates; Table 2, Table 3 and Supplementary Table 1). *P. solitum* has been found in cheese by several authors (Decontardi et al., 2018; Hocking and Faedo, 1992; Kure et al., 2004; Kure and Skaar, 2000; Lund et al., 1995). Our isolates were not producers of any of the three mycotoxins assessed (Table 3), which is in agreement with the characteristic of the species (production of mycotoxins unknown) (Frisvad and Samson, 2004). In addition, the Ehrlich reaction was negative, and the reaction on creatine was acidic. The reverse on YES agar was yellow-orange. Microscopically, the isolates had rough-walled stipes and globose to subglobose smooth-walled conidia. No growth at CYA/30 °C was observed (Table 3). As already mentioned, ITS sequencing was of no use in differentiating between *P. solitum* and *P. commune*, but *BenA* barcoding clearly confirmed the identification (Supplementary Table 1 and Fig. 1 and Supplementary Fig. 1).

Two isolates were identified as *P. chrysogenum*. The main features that led us to this identification were the ornamentation of the stipes (smooth; this is the only one with this characteristic among the species associated with food), the ability to grow well on CYA at 30 °C and the inability to produce the three extrolites tested (CPA, OTA, and PAT) (Tables 1 and 3). ITS sequencing identified the isolates as *P. rubens* (Supplementary Table 1), a synonym of *P. chrysogenum* (Frisvad and Samson, 2004), and once again *BenA* sequence analysis correctly identified them, even though the use of this molecular marker with suspected isolates of *P. chrysogenum* should be carried out with care. It should be considered that *P. chrysogenum* is a regular cheese spoiler (Aran and Eke, 1987; Barrios et al., 1998; Frisvad and Samson, 2004; Hayaloglu and Kirbag, 2007; Hocking, 1994; López-Díaz et al., 1995; Lund et al., 1995). *P. chrysogenum* produces penicillin and several mycotoxins, some of which -for example, roquefortine- have been detected in cheese (Finoli et al., 2001; Kokkonen et al., 2005; López-Díaz et al., 1996).

In our study, isolate M32 was the only OTA producer and was identified as *P. nordicum.* There are only two species associated with cheese-spoiling producers of OTA, *P. nordicum* and *P. verrucosum* (Table 1). Phenotypically, they are differentiated from each other based on the cream/yellow reverse on YES agar for *P. nordicum* and on the red-brown reverse for *P. verrucosum* (Frisvad and Samson, 2004; Larsen et al., 2001) (Table 3) (this difference was clearly seen between strain M32 and the reference strain of *P. verrucosum* CECT, 20766). *P. nordicum* is generally associated with high-protein foods such as cheese, while *P. verrucosum* is more common in cereal products and other plant sources, although it has been isolated from cheese as well (Hocking and Faedo, 1992; Larsen et al., 2001). The identification of M32 by ITS sequencing yielded an inconclusive result (Supplementary Fig. 1), whereas *BenA* analysis correctly identified it as *P. nordicum*, although it is very close to *P. verrucosum* (Fig. 1).

OTA is a nephrotoxin that affects all tested animal species, though effects on humans have been difficult to establish unequivocally (Perrone and Susca, 2017). It is listed as a “possibly human carcinogen” (Class 2B) (IARC, 1993). OTA has been found in cheese by several authors (Anelli et al., 2019; Dall’Asta et al., 2008; El-Sawi et al., 1994; Engel, 2000; Jarvis, 1983; Sinha and Ranjan, 1991). This mycotoxin is fairly stable in cheese, and Coton et al. (2019) demonstrated its production and migration up to 1.6 cm in depth, but current regulation in the European Union excludes cheese from the foodstuffs with maximum levels of OTA (Bullerman, 1981; European Commission, 2006).

One isolate was identified as *P. expansum*. Morphologically, it is one of the few species of the subgenus *Penicillium* isolated from cheese with smooth stipes, and another of its characteristics is a strongly violet reaction with Ehrlich reagent and the ellipsoidal conidia (Tables 2 and 3). Our strain was positive for production of patulin, which is also typical for this species (Table 3) (Pitt and Hocking, 2009) (Table 3). Patulin has also been found in cheese (Lafont et al., 1979), but current regulation in the European Union excludes cheese from the foodstuffs with maximum levels of PAT (European Commission, 2006).

Finally, one isolate was found to be *P. cvjetkovicii*. The species, belonging to section *Cinnamopurpurea*, subgenus *Aspergilloides*, has been described very recently (Peterson et al., 2015). Identification was performed initially by ITS and *BenA* sequencing, which led to inconclusive results, due to the low number of sequences available in genetic databases (Supplementary Table 1). Final identification was confirmed by morphological features and calmodulin *CaM* gene analysis. Morphologically, this fungus is characterized by the *monoverticillata* penicilli and the production of vinaceous to reddish-brown soluble pigments. A cheese isolate obtained from Spain (Marín et al., 2014) was identified by Peterson et al. (2015) as *P. cvjetkovicii*, although it had initially been considered to be *P. chermesinum* (Marín et al., 2014; Peterson et al., 2015). The isolate obtained in our work would be the second finding of it in cheese. In our case, the fungus produced a spoilage on the surface of ripened cheeses (Castellano) that was characterized by small dark spots. The spoilage occurred in one factory, and the probable origin was the air (Peterson et al., 2015).

According to the results of other authors and taking into account the results of our study, there are some secondary metabolites produced by the *Penicillium* isolates associated with cheese that would be worth investigating to help with identification—for example, CPA. With a simple technique such as the agar plug described by Samson et al. (2010) and used in our study, it is possible to discard 12 out of 15 species associated with cheese, which is very useful in the absence of molecular techniques (as mentioned before, *P. commune* is the most frequent *Penicillium* in ripened cheeses and also found to be dominant in our study).

Regarding the molecular analysis, the limitations of ITS as a species marker for differentiation of *Penicillium* species were clearly demonstrated, as no isolate, except *P. expansum*, was unequivocally identified by this procedure. The use of the secondary marker *BenA,* as proposed by Visagie et al. (2014), was useful for establishing the distinction between *P. commune* and *P. solitum*, which was not resolved by ITS (although they can easily be differentiated by testing CPA production), as well as that between some species that are very difficult to identify using just phenotypic characteristics (*P. commune* and *P. palitans* or *P. nordicum* and *P. verrucosum*). A third marker, the *CaM* gene, had to be used in this work to ensure the identification of the unexpected finding of the newly described species *P. cvjetkovicii*.

In conclusion, the results of this study indicate the presence of spoilage and mycotoxigenic species dominated by *P. commune* on the surface of the cheeses investigated. The presence of mycotoxigenic moulds on the surface of cheese is of concern, as several authors have detected CPA, OTA, and PAT in cheese. Although it is unclear whether the levels of the mycotoxins found could be harmful for the consumer, the fact is that the isolates found in our study are mostly mycotoxigenic, and their ability to contaminate cheese should be considered in order to define potential health risks.

Nevertheless, the role of *P. commune* in cheese ripening remains to be determined, as some authors claim that it has a positive contribution to the sensory characteristics of cheese. This role is under study by the authors at present.

The GenBank accession numbers for the *BenA* gene and ITS sequences of the 32 isolates used in this study are MK675757-MK675788 and MK660326-MK660357 respectively, and those of the *CaM* gene sequences of the isolates Q2M7 and Q3M1 are MK660604-MK660605.

The strains of *P. commune* M35 and *P. nordicum* M32 are available from the Spanish Type Culture Collection as CECT 20940 and CECT 20939, respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103253.

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