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Fungal mycobiota and mycotoxin risk for traditional artisan Italian cave cheese

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ABSTRACT

Cave cheese is a surface mold-ripened variety of cheese produced also in South of Italy, exploiting fungal population naturally occurring on cave walls, as part of secondary microbiota for ripening. In this study, 148 fungal strains were isolated from 22 independent cave cheese samples, collected in 13 Italian geographical locations, mostly in Apulian area.

DNA-based identification showed the presence of twenty-four fungal species in the outer part of the cheese ripened in caves. *Aspergillus westerdijkiae* and *Penicillium biforme* resulted the most frequently isolated species, followed by *Penicillium roqueforti* and *Penicillium solitum*. The 86% of cheese sample presented at least one toxigenic species and the 45% revealed the presence of ochratoxigenic species, *A. westerdijkiae* and *A. steynii*, suggesting possible mycotoxin risk during ripening stage in caves, confirmed by the presence of ochratoxin A (OTA) in the rind of 36% of samples.

In conclusion, cave cheese is a susceptible product for toxigenic mold growth and in particular OTA contamination, therefore adeguate scientific tools for matching organolectic consumer expectations and complete safety of food should be developed, as well as spontaneously molded and not monitored cheeses should not be consumed to avoid mycotoxin risk.

1. Introduction

Cave cheese is a traditional Italian semi-hard cheese, commonly produced and consumed in Apulian area, made from raw milk of sheep or cow and aged in natural cellars or tufa caves, typically warm, moist and moldy environments. The use of fungi in food production, as part of secondary microbiota contributing to the food processing and organoleptic properties, is common due to their critical role in adding, modifying or removing components, including flavors, nutritional elements such as vitamins or colors to enhance the appeal of the food (McSweeney, 2004; Fox et al., 2017).

Ripening of cave cheese typically take place in caves, where temperature is 15–25 °C and humidity 85–90%, as reported by cheesemakers, exploiting fungal population naturally occurring on cave walls, without adding any fungal starter culture. Autochthon molds spontaneously colonize cheeses during the ripening and attribute rheological and sensorial characteristics, very much appreciated by the consumers. Caves specific mycobiota is composed of *Aspergillus* and *Penicillium* species, including toxigenic species (Creppy, 2002; Erdogan et al., 2003; Castegnaro et al., 2006; De Santi et al., 2010; El-Fadaly et al., 2015; Fontaine et al., 2015; Decontardi et al., 2017), possibly affecting safety of food products with mycotoxins, well-known to be carcinogenic or genotoxic to both animals and humans. The fungal population is considered by cheesemaker as an added value for cheese, linked with land and therefore able to make product unique and typical, supposed to be characterized by solid level of quality, and therefore often more expensive than equivalent products, made by large scale and controlled production. At the end of maturation process, cheeses are usually washed and brushed, to remove the moldy residues developed on the surface, eliminating information about grown fungal population.

Mycotoxigenic fungi can occur on cheese and can grow in conditions comparable to ones characterizing caves used for ripening, arising the possible multitoxin contamination risk (Camardo Leggieri et al., 2016). Several worldwide studies detect fungal secondary metabolites in cheeses, mostly citrinin, penitrem A, roquefortine C, sterigmatocystin and aflatoxin and ochratoxin A (OTA) (Creppy, 2002; Erdogan et al., 2003; Sengun et al., 2008; Ostry et al., 2013; Pattono et al., 2013; Båth et al., 2012; Fontaine et al., 2015; Dobson, 2017; Decontardi et al.,

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2017; Kalinina et al., 2018), with or without characterize mycobiota occurring on them, as well as other studies report presence of toxigenic species on cheese surfaces, without measuring possible mycotoxins content in cheeses (El-Fadaly et al., 2015; De Santi et al., 2010; Yuvaşen et al., 2018). Currently, the EU General Food Law and worldwide other organization currently do not regulate the mycotoxin in cheese, except for aflatoxin M_1 (AFM1AFM1), which occurs in the milk of lactating animals as hydroxylated derivative of aflatoxin B_1 (AFB1AFB1).

The aim of the present study was to survey the fungal community spontaneously colonizing ripening cave cheese in artisan production and relate it to possible mycotoxigenic risk. Characterization of mycobiota was set up combining morphological and molecular identification of strains, evaluating their possible ability to produce mycotoxins "*in vitro*", as well as measuring possible mycotoxins content directly in cheese.

2. Materials and methods

2.1. Sampling of cheese

Twenty-two samples of cave ripened cheeses, mostly spherical with diameter in the range from 15 cm to 20 cm, were bought directly from cheesemakers or dairy industry stores, mainly in Apulian area. Some of them were bought after been washing, some others collected from the ripening caves or cellars, with a visible fungal growth. Some available information about features of cheese samples were collected from the cheesemakers or the related production protocols and are reported in Table 1.

Table 1
Sample's information for cheeses included in the study.

Sample	Origin Town (province)*	Surface aspect	Raw material	Ripening period
A	Gravina (BA)	NB, NW	Cow's milk	9 months
B	Gravina (BA)	B, W	Cow's milk	9 months
C	Biccari (FG)	NB, NW	Sheep's milk	2 months
D	Gravina (BA)	B, W	Cow's milk	7months
E	Santeramo (BA)	B, W	Cow's milk	18 months
F	Santeramo (BA)	NB, NW	Cow's milk	6 months
G	Altamura (BA)	NB, NW	Cow's milk	3 months
H	Altamura (BA)	NB, NW	Cow's milk	18 months
J	Biccari (FG)	NB, NW, Walnut Leafs On The Surface	Goat's milk	n.a.
K	Martina Franca (TA)	NB, NW	Sheeps and Cow's milk	n.a
L	Martina Franca (TA)	B, W	Cow's milk	18 months
M	Martina Franca (TA)	NB, NW	Cow's milk	11 months
N	Martina Franca (TA)	NB, NW	Cow's milk	n.a.
O	Martina Franca (TA)	B, W	Sheeps and Cow's milk	12 months
P	Martina Franca (TA)	B, W	Sheeps and Cow's milk	18 months
R	Biccari (FG)	NB, NW	Goat's milk	n.a.
S	Torino	B, W	Cow's milk	n.a.
T	Turi-Putignano (BA)	NB, NW	Cow's milk	n.a.
U	Cuneo	B, W	Sheep, Goat and Cow's milk	2 months
V	Biccari (FG)	NB, NW	Goat's milk	n.a.
W	Zungoli (AV)	NB, NW	Cow's milk	n.a.
Z	Biccari (FG)	NB, NW, Olive Tree Leafs On The Surface	Sheeps and Cow's milk	n.a.

NB= Not Brushed; NW= Not Washed; B= Brushed; W= Washed; n.a. = not available; *:BA= Bari, FG= Foggia, TA = Taranto, AV = Avellino.

2.2. Isolation of fungal strains

Fungal sampling from cheese was conducted plating 3 replicates of rind pieces (obtained with a cork borer with 1.5 cm diamenter) on Dichloran Rose-Bengal Chloramphenicol (DRBC) (King et al., 1979) agar media at 28 °C for 5–7 days, to isolate mycobiota growing on outside layer of cheese. Subsequently colonies were transferred on Potato Dextrose Agar (PDA) for pure cultures production, and subsequently on Czapeck Dox modified agar medium for monosporic isolation.

Fungal genera and species were firstly determined observing macroscopic and microscopic features of colonies according to the taxonomic keys of Klich (2002) and Samson et al (2010): the colony diameter, color (conidia and reverse), exudates and colony texture, as well as conidial heads, stripes, color and length vesicles shape and seriation, metula covering, conidia size, shape and roughness. Each strain was stored in 20% glycerol solution at −80 °C. The 22 cheese samples included in the study yielded more than 2000 yeast and fungal colonies. A subset of 158 isolates was obtained in pure culture and only 148 were selected, according to morphological features, as representative mold-like population occurring on samples, and subsequently submitted to DNA isolation and sequencing.

2.3. DNA-based identification of fungi

The DNA-based identification of fungi was carried out on a subset of 148 representative fungi isolated from cheeses. 100 µl (10^6/mL) of conidia suspension obtained were spread on cellophane film stretch out on PDA, and incubated at 25 °C for 2 days in darkness. After incubation, mycelia grown on cellophane film, to facilitate the mycelium scraping and get slower conidia's production, was collected in tubes with a scraper and used for DNA isolation. DNA isolation was obtained using the Wizard® Magnetic Purification System for Food" kit (Promega, USA) starting from 30 to 40 mg of freezed ground mycelium. Quality and yield of resulting DNA were evaluated by agarose gel electrophoresis. In the present survey, a first identification was performed using Internal transcribed spacer (ITS), the most commonly sequenced gene for fungi and recently accepted as the official DNA barcode (Schoch et al., 2012). However, it often does not provides enough information for species identification (Skouboe et al., 1999; Peterson, 2000a; Peterson, 2000b; Samson et al., 2011). In order to compensate the lack of variability in ribosomal internal transcribed spacer (*ITS*), we used also beta tubulin gene (*benA*), as identification marker, and in the cases of *Aspergillus versicolor* section also RNA polymerase II second largest subunit (*rpb2*). DNAs amplification was set up for Internal transcribed spacer (*ITS*) with ITS4/ITS5 primers (White et al., 1990) and for β-tubulin gene (*benA*) with BT2a/BT2b primers (Glass and Donaldson, 1995), a key gene in the taxonomy of *Aspergillus* (Kocsubé et al., 2016) and *Penicillium* (Visagie et al., 2014). In some cases, RPB2 gene (*RNA polymerase II second largest subunit*) was also sequenced with 5F/7CR primers (Liu et al., 1999), according to Samson et al. (2014), in particular to discriminate species belonging to *Versicolores* section (like *A. tennesseensis*, *A. jensenii*, *A. creber, A. puulaauensis, A. cvjetkovicii*). After amplification, amplicons were purified with the enzymatic mixture EXO/SAP (Exonuclease I, *E. coli*/Shrimp Alkaline Phosphatase) and used as template for bidirectional DNA sequencing. Sequencing was performed with the BigDye v3.1 terminator kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction and analyzed on an ABI 3730 XL Genetic Analyzer (Applied Biosystems). Alignment of the two strands was performed using the software package BioNumerics 5.1 (Applied Maths), with manual adjustments where necessary, evaluated by an experienced human eye. Beta tubulin (135) and RPB2 (15) sequences were deposited in the European Nucleotide Archive (ENA) with following accession numbers: LS423454-LS423603.

Species identifications were performed for each strain using the BLAST algorithm (http://www.ncbi.nlm.nih.gov), against the non-

redundant database maintained by the National Center for Biotechnology Information.

2.4. Phylogenetic analysis

To infer phylogenetic relationships, nucleotide sequences of housekeeping genes (*benA* and *rpb2*) were aligned with ClustalW and then subjected to maximum parsimony analyses with MEGA version 7.0 (Tamura et al., 2011), including type- or reference-species strains. All positions containing gaps and missing data were eliminated from the analyses. Statistical significance of branches in the resulting trees was assessed by bootstrap analysis using 500 or 1000 pseudoreplications (Felsenstein, 1985).

2.5. Mycotoxins production in vitro

Fungal mycotoxins production was investigated in relation to the possible toxigenic profile reported in literature for the assigned species. A subset of 48 fungal strains, representative of species isolated from each cheese, were cultured on yeast extract sucrose agar (YES) (Frisvad and Samson, 2004), for 14 days at 25 °C in darkness. For the 48 stains sub set, at least 1 isolate per species was selected and multiple isolates for *A. westerdijikae*, due to the know species composition made by OTA producers and non producers strains (Morello et al., 2007).

Determination of mycotoxins in fungal cultures. Mycotoxins stock were reconstituted with appropriate solutions: OTA with acetonitrile/water/acetic acid (99:99:2, v/v/v), PAT with water/acetonitrile (85:15, v/v), AFB1 and AFB2 with water/methanol (60:40, v/v), STC with water/acetonitrile (75:25, v/v), CIT with acetonitrile/water/acetic acid (99:99:2, v/v/v). Appropriate ranges of standard solutions were then obtained: 50–100 ng/mL (OTA), 80–800 ng/mL (PAT), 0.4–10.0 ng/mL (AFB1), 0.08–1.4 ng/mL (AFB2), 500–5000 ng/mL (STC), and 100–2000 ng/mL (CIT). 1 g of agar with fungal culture was extracted with 5 mL of acetonitrile/methanol/water (90:90:80, v/v/v) for OTA, PAT and CIT, and with 5 mL of methanol/water (80:20, v/v) for AFB1, AFB2 and STC, on orbital shaker for 60 min. Residues were dissolved in 1 mL of appropriate solutions and filtered using RC 0.20 μm regenerated cellulose filter: acetonitrile/water/glacial acetic acid (99:99:2, v/v/v) for OTA and CIT, water/acetonitrile (85:15, v/v) for PAT, water for AFB1 and AFB2, water/acetonitrile (75:25, v/v) for STC. 50 μl of extracts was injected into to HPLC apparatus (Agilent 1260 Series, Agilent Technology, Santa Clara, CA, USA). The quantification of mycotoxins were performed by HPLC/FLD according to Susca et al. (2016) for OTA, Sewram et al. (2000) for PAT, Fani et al. (2014) for AFBs, Veršilovskis et al. (2008) for STC and Lee et al. (2006) for CIT. The detection limits, based on a signal to noise ratio of 3:1, were as following: 2 μg/kg (OTA), 5 μg/kg (PAT), 0.2 μg/kg (AFB1), 0.04 μg/kg (AFB2), 4 μg/kg (STC), 5 μg/kg (CIT).

2.6. Mycotoxins in cheese samples

Each cheese was cut according scheme in Fig. 1, to obtain various subsamples representative of different cheese areas, from the rind to the inner part: X-1-0 (rind and at least 2 cm of part next to it), X-1-1, and X-1-2 (cheese center). Slices and subsamples were named using a letter (here generically named X), indicating the cheese sample, and numbers indicating slices and subsamples, so keep information about area of origin in the cheese.

Mycotoxins were analyzed according to published protocols with slight modifications: OTA according to C. Dall'Asta et al. (2008), PAT was analyzed according to CEN-prEN 14177 (2003), AFM1 according to S. Dragacci et al. (1995), STC and AfB1 according to AOAC Official Method 974.16 B Method II (2000) with HPLC determination. 5 g of cave cheese was homogenized with 5 mL of water by Ultraturrax (T50 basic IKA®-WERKE, Staufen, Germany) for determination of OTA, PAT, AFM1, with 5 mL of methanol/water (55:45, v/v) for determination of STC, AFB_1. Mycotoxins levels were measured comparing peak areas with calibration curve obtained with respective mycotoxin standard solution. The detection limits, based on a signal to noise ratio of 3:1, were as following: 0.1 μg/kg (OTA), 0.5 μg/kg (PAT), 0.4 μg/kg (AFM1), 0.5 μg/kg (STC), and 0.1 μg/kg (AFB1).

Determination of Ochratoxin A. 5 g of slurry was extract with 50 mL of CH_2CL_2 plus 0.75 mL of 85% H_3PO_4 (pH = 3) and 1 mL of NaCl satured solution on an orbital shaker for 120 min. The extract was filtered through filter paper Whatman n° 4 and extracted two times with 10 mL of NaCl satured solution. A volume of aqueous extract (10 mL) was cleaned up through an OchraTest immunoaffinity column (Vicam, Watertown, MA) and quantified using (HPLC/FLD) according method described in Cozzi et al. (2013).

Determination of Patulin. 5 g of slurry were weighted and 30 mL of ethyl acetate were added, vortex 2 min, centrifuge at 4500 rpm 5 min and transfer the supernatant to another flask (x 3 times). 10 mL of Na_2CO_3 (1.5% in water) were added to organic phase (90 mL), after shaking during 1 min. The aqueous phase was separated and the organic phase was filtered using filter paper Whatman n° 4 with sodium anhydrous sulphate. The filtered was concentrated under an air stream at ca. 50 °C to reduce the volume. Then 2 mL of acetic acid in water (pH = 4) and 2 mL of hexane were added mix 1 min 500 μL of aqueous phase were filtered using RC 0.20 μm regenerated cellulose filter. 50 μl of filtered was injected into to HPLC apparatus. The quantification of Patulin by HPLC/DAD is described in Sewram et al. (2000). The mean recovery obtained from spiked samples in triplicate at 50 μg/kg was 78% with RSD < 10%.

Determination of Aflatoxin M_1. Mycotoxin stock solution of AFM1 (10 μg/mL) was prepared by dissolving solid commercial toxins in acetonitrile. Aliquots of the stock solution were transferred to 4 mL amber silanized glass vials and evaporated to dryness under a stream of nitrogen at 50 °C. The residue was dissolved with water/acetonitrile (75:25, v/v) to obtain calibrant standard solutions from 1.0 to 10.0 ng/mL 5 g of cave cheese was homogenized with 5 mL of water by Ultraturrax. 5 g of slurry were analyzed according to S. Dragacci et al. (1995). 50 μl was injected into to HPLC apparatus. The quantification by HPLC/FLD is described in Loi et al. (2016).

Determination of Sterigmatocystin and Aflatoxin B_1. STC and AFB_1 were analyzed according to AOAC Official Method 974.16 B Method II (2000) with HPLC determination. 5 g of cave cheese was homogenizedby blending with 50 mL methanol/water (55:45, v/v), 20 mL n-hexane and 2 g of NaCl, for 2 min. The extract was filtered through filter paper Whatman n° 4, collect 10 mL aqueous methanol phase add 10 mL CH_2CL_2 and shake by 1 min (×2) recombine CH_2CL_2 phase and 10 mL evaporate under an air stream at ca. 50 °C and reconstitute with 500 μL of the HPLC mobile phase. STC was determined using an analytical method reported by Veršilovskis et al., 2008. The analysis of the AFB_1 have been performed using the following analytical method (Fani et al., 2014).

3. Results

3.1. Isolation and identification of fungal population

In some cases, also *Mucor* spp. were isolated, but they were not further identified because considered not characteristic for mycobiota of cave cheese.

A preliminary DNA based identification was performed with ITS gene, to confirm uncertain morphological based identification at genus level. BLAST analysis (NCBI, 2017) for species identification was indeed performed analyzing sequence of beta-tubulin gene (*benA*) for all 148 strains (Table 2).

The most part of identified fungal specis belonged to potentially toxigenic genera: *Aspergilllus* (13), *Penicillium* (10) and *Alternaria* (1). Seven isolates of following species, *Microascus melanosporus*, *Neosartorya hiratsuka*, *Scopulariopsis brevicaulis*, *Scopulariopsis flava*,

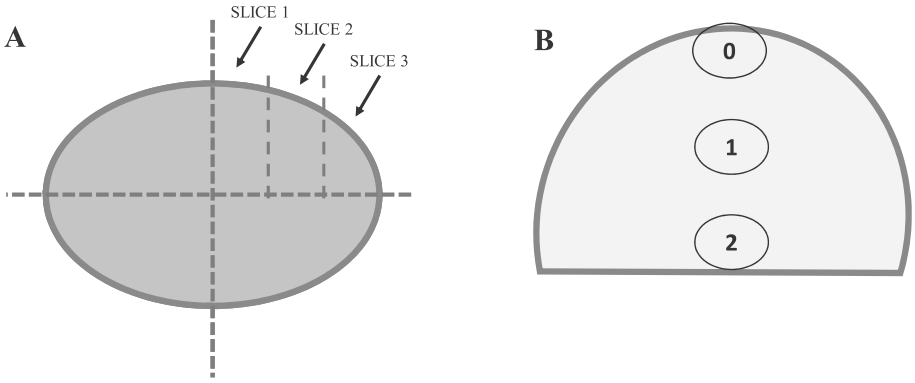


Fig. 1. Cheese sampling scheme.
Each cheese was divided in 4 parts, drawing perpendicular diagonals; each quarter was cut in 3 slices 1-2-3 (A), and each slice was used to obtain further 3 subsamples 0, 1, 2 (B).

Cladosporium sphaerospermum, were also collected, consistently with sporadic occurrence on cheese reported in literature (Banjara et al., 2015; Montagna et al., 2004; Ropars et al., 2012).

Initially, as shown in Table 2, a correct identification was not possible for 5 strains isolated from sample 1, because the BLAST search (NCBI, 2017) showed for *benA* 88% as maximum similarity to *P. cinnamopurpureum* with a lot of hits at 84–87% to clearly unrelated species of *Penicillium*, and subsequently it was described as a new species, named *Penicillium gravinicasei* (Anelli et al., 2018; MG600656) belonging to *Penicillium* section *Cinnamopurpurea.*

Among *Penicillium* and *Aspergillus* species, *P. jugoslavicum, P. salamii, P. solitum, P. gravinicasei*, *A. pseudoglaucus* and *A. tritici*, do not produce any type of mycotoxins, as reported in literature (Perrone et al., 2015; Decontardi et al., 2017; Anelli et al., 2018) therefore any mycotoxin analysis has been performed on the species.

Other species able to produce mycotoxins were isolated (Table 2), among which *A. westerdijikae* and *A. steynii* caught our attention, because OTA producers (Frisvad et al., 2004; Gil-Serna et al., 2011, 2015; Morello et al., 2007).

Among *Aspergillus* species isolated, 15 strains belonging to Section *Versicolores*, showed the same percentage of similarity with multiple species, suggesting an additional DNA marker sequencing for correct identification (Samson et al., 2014). It was the case of *A. amoenus, A. creber, A. cvjetkovicii, A. puulaauensis, A. jensenii, A. tennesseensis,* reported in literature as possible sterigmatocystin producers (Jurjević et al., 2013). Only one strain was identified as *A. flavus*, potentially AFBs producer.

In *Penicillum* genus, the most spread species resulted *P. biforme,* in the past considered as synonymous of *Penicillium camemberti* and *P. commune* (Frisvad and Samson, 2004), but recently defined as distinct species (Giraud et al., 2010), strongly associated with cheese and related environments.

Other species isolated in at least 1 cheese sample were *P. paneum*, know as patulin producer (Frisvad et al., 2004; Varga et al., 2015); *P. flavigenum*, *P. paneum, P. roqueforti, P. chrysogenum,* and *P. crustosum* as roquefortine C producers (Frisvad et al., 2004; Varga et al., 2015); *A. alternata* as producer of AAL-toxin (Varga et al., 2015).

The distribution of fungal species in the recruited samples (Fig. 2) revealed from 1 to 8 distinct fungal species in any individual cheese and the largest number of species was detected in cheese samples “K” (8) followed by “T” (7) and “W” (6). Two species showed broaden distribution, *A. westerdijikae* (10/22) and *P. biforme* (16/22), spread on 13 different cheese samples, and presumably in related caves.

3.2. Phylogenetic analysis

Phylogenetic relationship were also analyzed to solve umbiguities obtained with BLAST approach for *Penicillium* Sect. Viridicata Ser. Camemberti spp. as *P. commune* and *P. biforme*, and *Aspergillus* Sect. *Versicolores* spp. and to evaluate possible intra-species nucleotide variability. After the trimming and cleaning operations, they were assembled and aligned according to the CLUSTAL W method. The evolutionary relationships between the strains were obtained by the method of Maximum Parsimony (MP) and are showed in Fig. 3 (A) for *Penicillium* genus, in Fig. 3 (B) for *Aspergillus* genus. Among DNA-based identification, 54 out of 62 in *Aspergillus* genus and 73 out of 78 strains in *Penicillium* genus confirmed the results obtained initially with BLAST, clustering with related species reference strains retrieved from GenBank (Visagie et al., 2014; Kocsubé et al., 2016; Giraud et al., 2010).

The phylogenetic analysis of *benA* resolved 33 isolated of *P. biforme*, and none strain belonging to *P. commune* or *P. caseifulvum* or *P. camemberti*. To clearly descrimante Sect. *Versicolores* species, *rpb2* gene was sequenced, reported as more informative then *benA* for those species according to Samson et al. (2010). The 15 isolates have been identified unequivocally through Genealogical concordance philogenetics species recognition method (GCPSR, Taylor et al., 2000), based on *rpb2* and *benA* (Fig. 4): 5 isolates clustered with *A. jenseni* (NRRL 58600), 2 with *A. tenessensis* (NRRL 12150), 1 strain with *A. amoenus* (NRRL 4838), 4 with *A. puulaauensis* (NRRL 35641), 1 with *A. venenatus* (NRRL 58592), 2 with *A. creber* (NRRL 58592). The presence of *Aspergillus* sect. *Versicolores* spp. arises possible risk of sterigmatocyste contamination in cheeses (Jurjević et al., 2013).

3.3. Mycotoxins production in vitro

A subset of 48 isolates, out of the 148 identified by DNA sequencing, were tested for their ability to produce mycotoxins “*in vitro*”, focusing analysis on species potentially producing EU regulated mycotoxins (aflatoxin M1 and B1, citrinin, ochratoxin A, sterigmatocystin and patulin), at least in other food and expected mycotoxins for species according to literature (Table 3).

Isolates of *A. westerdijkiae* collected in the survey resulted in a mixed population of OTA producing and OTA non-producing strains, under tested conditions. Isolates of *A. steynii* were collected only from two samples and all of them resulted able to produce OTA under tested conditions. *A. puulaauensis*, produced sterigmactocistin and also AFB1 confirming data from Jurjević et al. (2013). Finally, the unique strains of *A. flavus* isolated was able to produce *in vitro* both AFB1 and AFB2, as reported by Varga et al. (2015).

3.4. Occurrence of mycotoxin in cheese

Mycotoxins investigations in cheese (Table 4) were conducted in relation to the potential toxigenicity associated to the fungal species isolated from the sample (Table 2).

OTA presence was tested in all cheese samples, due to the wide

Table 2
Results of BLAST analysis for 148 isolates fom cheese, using *BenA* or *rpb2* gene.

N° isolates	N° contaminated cheese	Similarity (%)	*BenA* Best match (BLAST)[a]	*rpb2* Best match (BLAST)[a]	Possible mycotoxin for the species
33	15	100%	– *P. biforme CBS 297.48 (FJ930944)* – *P. commune CBS31148 (AY674366)* – *P. caseifulvum CBS 101134 (AY674372)* – *P. camemberti MUCL29790 (FJ930956)*		Rugulovasine A & B29 [b,c]
1	1	99%	*P. chrysogenum CBS 306.48 (AY495981)*		**Roquefortine C** [b]
1	1	100%	*P. crustosum CBS 101025 (AY674351)*		**Roquefortine C** [b]
1	1	99%	*P. flavigenum CBS 419.89 (AY495991)*		**Roquefortine C** [b]
6	1	98–100%	*P. jugoslavicum CBS 192.87 (KC773789)*		Unknown
2	1	100%	*P. paneum CBS 101032 (AY674387)*		**Patulin** [1−3], Botryodiploidin, Roquefortine C [b]
11	5	99–100%	*P. roquefortii CBS 22130 (AY674383)*		Mycophenolic acid, Roquefortine C [b]
1	1	100%	*P. salamii ITEM 15291 (HG514437)*		None [e]
17	7	98–99%	*P. solitum CBS 42489 (AY674354)*		None [f]
5	1	88%	*P. cinnamopurpureum NRRL 35502 (EF506216)*		None [g]
1	1	100%	*A. alternata CS36-5 (KY814630)*		Fumonisins like called AAL-toxin [d]
1	1	100%	*A. amoenus NRRL 4838 (EF652304)*	*A. amoenus NRRL 4838 (JN853824)*	**Sterigmatocystin** [h]
2	1	98–100%	*A. candidus NRRL 58959 (LT626989)*		None[i]
2	2	99%	*A. creber NRRL 58592 (JN853980)*	*A. creber NRRL 58592 (JN853832)*	**Sterigmatocystin** [h]
1	1	100%	*A. flavus isolate CICC 40184 (KX462752)*		**Aflatoxins B1 & B2**, cyclopiazonic acid, kojic acid, aspergillic acid [d]
8	5	100%	– *A. jensenii NRRL 58600 (JN854007)* – *A. tennesseensis s NRRL 13150 (JN853976)* – *A. cvjetkovicii NRRL 227 (EF652264)*	– *A. jensenii NRRL 58600 (JN853835)* – *A. tenessensis NRRL 13150(JN853806)* – *A. cvjetkovicii NRRL 227(EF652176)* – *A. venenatus NRRL 13147 (JN853832)*	**Sterigmatocystin** [h, j]
1	1	99%	*A. pseudoglaucus NRRL 40 (EF651917)*		Unknown
4	4	100%	*A. puulaauensis NRRL 35641 (JN853979)*	*A. puulaauensis NRRL 35641 (N853823)*	**Sterigmatocystin** [h]
7	2	100%	*A. steynii NRRL 35675 (EF661347)*		**OTA**, Penicillic acid [b,d]
2	1	99%	*A. tritici CBS 26681 (KP987051)*		Unknown
34	9	100%	*A. westerdijkiae ITAL 234 (EF150881)*		**OTA**, Penicillic acid [b,d]
1	1	100%	*Microascus melanosporus DTO 255-C3 (KX924339)*		Unknown
1	1	100%	*Neosartorya hiratsukae UOA/HCPF EM9708 (FJ433874)*		Unknown
1	1	99%	*Scopulariopsis brevicaulis DTO 148H5 (JQ434543)*		Unknown
3	2	99%	*S. flava CBS 207.61 (KX924457)*		Unknown
1	1	99%	*Cladosporium sphaerospermum EXF-1061 (EF101408)*		Unknown

Unknow = any study available.
[a] GenBank Accession N° are in brackets.
[b] Frisvad et al., 2004.
[c] Giraud et al., 2010.
[d] Varga et al, 2015.
[e] Perrone et al., 2015.
[f] Decontardi et al., 2017.
[g] Anelli et al., 2018.
[h] Jurjević et al., 2013.
[i] Elaasser et al., 2017.
[j] Despot et al., 2016.

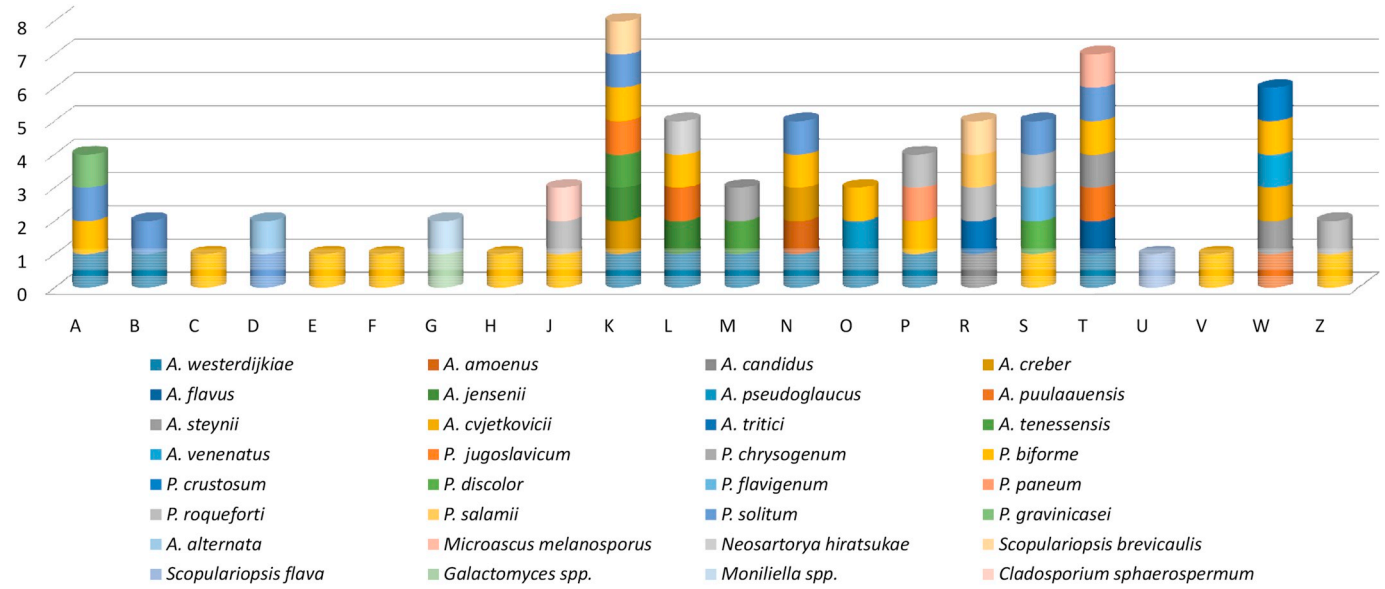


Fig. 2. The distribution of fungal species in the recruited cheese samples.
Mycobiota naturally occurring on cave cheese from South of Italy. The survey revealed *A. westerdijikae* (10/22) and *P. biforme* (16/22) the most occurring species on cheese samples from the 13 different dairies.

distribution of *Aspergillus* ochratoxigenic species. OTA was detected in 8 cheese samples, in the range from 317 μg/kg (sample "A") to 0.2 μg/kg (samples "P" and "W"), analysing a portion of rind, reported as edible in some cases. Splitting up brushed/washed (8/22) and moldy (14/22) rinds, data showed OTA contamination in the 25% of brushed/washed cheeses, in a range of 0,2 ÷ 49,08 μg/kg, and in the 50% of not brushed/washed cheeses, in a range of 0.2 ÷ 317.07 μg/kg. All tested samples representing center and parts immediately next to it (corresponding to subsamples from A-1-1 to Z-1-1 and from A-1-2 to Z-1-2) resulted negative to OTA determination. Aflatoxin M_1 and B_1, sterigmatocystin and patulin were not detected in any case.

Two samples ("A" and "B") with the higher OTA contamination, were further tested to evaluate possible OTA diffusion from rind to the center of cheese. The 2 samples were retrieved from the same cheesemaker, produced with the same raw materials (cow's milk), ripened in the same environment for nine months, resulted colonized by OTA producing strains of *A. westerdijkiae,* and differed only for external aspect of rind, not brushed/not washed for sample "A" and brushed/washed for sample "B" at collection time. Results showed higher OTA levels in sample "A" then "B" (Table 5), maybe due to the presence of mycelia which are the main mycotoxins holder. Looking at subsamples and referring to Fig. 1, we can backtrack the areas of subsamples origin, deducing an irregular distribution of OTA contamination in the rind, as well as, a sort of OTA gradient decreasing from different areas of the rinds (A-1-0, A-2-0, A-3-0, or B-1-0, B-2-0, B-3-0), to middle (A-1-1, A-2-1, A-3-1, A-3-2 or B-1-1, B-2-1, B-3-1, B-3-2) and finally to the center (A-1-2, A-2-2 or B-1-2, B-2-2).

4. Discussion

This study is the first survey on mycobiota occurring on artisan Italian, mostly Apulian, cave cheeses, analyzing simultaneously fungal species, their mycotoxigenic potential *in vitro* and mycotoxin content in cheese products.

Currently, investigations of moldy food at artisan level are few, despite of the consumer's belief of its high quality level. The topic draw in further interest, due to new cheese making protocols during ripening period, which go through covering cheese surface with vegetable matrixes to improve sensorial and rheological characteristics of foods, which represent a potential carryover of toxigenic fungal population (pistachios, walnuts, oat, marc, dried grass, etc.). The microbial populations that develop on the surface of cheese can be quite complex depending on environments of ripening and preparing. Some cheeses have traditionally been aged in jars and earthenware vessels buried in sand and soil (Kamber, 2007) and/or in caves, which bring to the cheese diverse types of microorganisms (Torsvik and Øvreås, 2002; Hansel et al., 2008). Members of soil fungal genera such as *Alternaria, Chrysosporium*, *Geotrichum, Mucor,* and *Penicillium* are commonly found on cheese ripened in cave environments (Flórez et al., 2007).

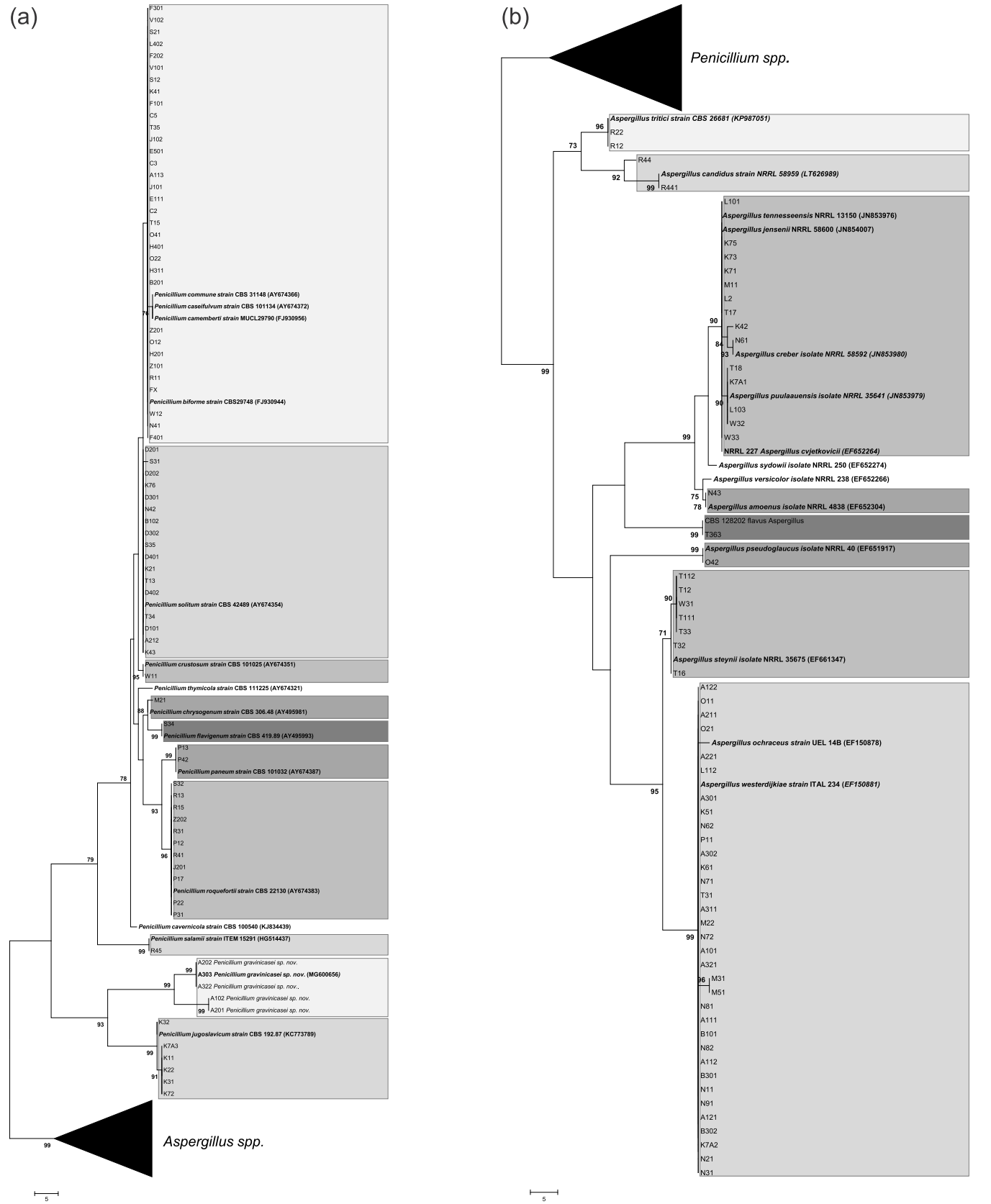
Growth of molds may be beneficial for the development of the characteristic flavor and aroma of dairy products, due to mold involvement with the degradation of lipids and proteins, and can also play a protective role against pathogenic or spoilage microorganisms, but at the same time they could be linked to potent contaminants, such as mycotoxins.

Data from literature (Erdogan et al., 2003; Budak et al., 2016; Dobson, 2017) draw attention to cave and cellar cheese as new sources of exposure to mycotoxins for health risk assessment. However, there is a lack of precise information about mycotoxin contents (JØrgensen, 2005; Heussner and Bingle, 2015), which makes incomplete and not adequate the "risk analysis" related to occurrence of mycotoxins and mycotoxigenic fungi in artisan cheese.

With the aim to study fungal population from the artisan cave cheese rind, crucial points are species recognition and strain typing, because of some fungal species can include toxigenic strains.

During cheese sampling and strains isolation, we rarely isolated fungal species commonly hosted in soil and grown on a wide range of substrate, as *Scopulariopsis flava* and *S. brevicaulis*, occasionally reported as opportunistic human pathogens (Hoog et al., 2000; Woudenberg et al., 2017); *M. melanosporus*; *Neosartorya hiratsuka* belonging to *Aspergillus* section *Fumigati; Cladosporium sphaerospermum*, reported as not mycotoxigenic fungi (Davis et al., 1975).

The main part of fungal population included *Aspergillus* and *Penicillium* species, with some toxigenic species, raising safety concerns, due to potential contamination by mycotoxins of chees rinds. *Aspergillus flavus*, was isolated from a single sample and is considered much more commonly associated with foods of plant origin. Aflatoxin in dairy is an acknowledged problem, but is associated with the metabolism of aflatoxin B1 in lactating cattle to produce the milk-associated aflatoxin M1, and not generally with the growth of fungi in dairy products. Actually,



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in a recent study, the maximum aflatoxin production on cheese based medium has been defined in the range 25–30 °C (Casquete et al., 2017), very close but different from common temperature in caves (15–25 °C). In fact, chemical analyses confirmed absence of M1 and AFB1 in all tested cheese samples.

The unique mycotoxin detected in the cheese sample was OTA and

Fig. 3. a - Dendrogram of *Penicillium* spp.
The tree shows the genetic relationships based on maximum parsimony (MP) method analyzing the partial b-tubulin gene dataset for *Penicillium* spp detected in the survey and for related type- or reference-species strains. The tree was rooted with grouped *Aspergillus* species identified in the study and showed in Fig. 3b. Numbers at nodes indicate support for the internal branches within the resulting trees obtained by bootstrap analysis.
b - Dendrogram of *Aspergillus spp*
The tree shows the genetic relationships based on maximum parsimony (MP) method analyzing the partial b-tubulin gene dataset for *Aspergillus* spp detected in the survey and related type- or reference-species strains. The tree was rooted with grouped *Penicillium* species identified in the study and showed in Fig. 3a. Numbers at nodes indicate support for the internal branches within the resulting trees obtained by bootstrap analysis.

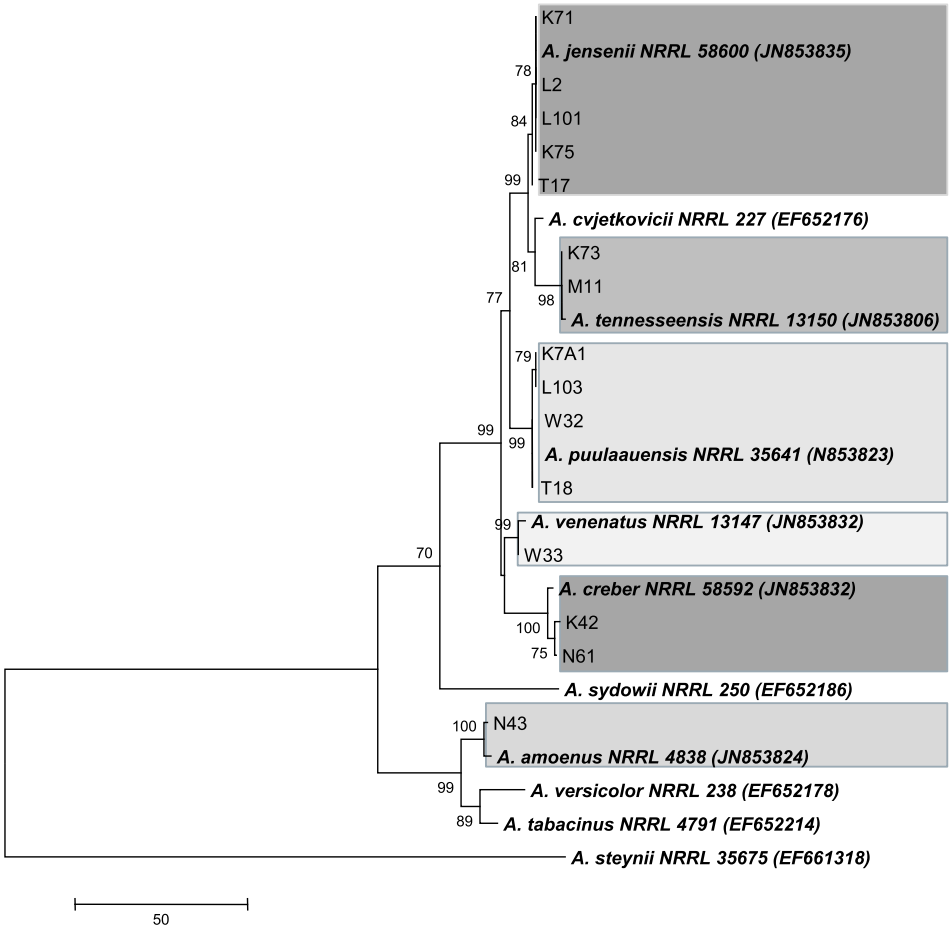

Fig. 4. Dendrogram *Aspergillus genus sec. Versicolores*
The tree shows the genetic relationships based on maximum parsimony (MP) method analyzing the partial *rpb2* and *benA* gene dataset for *Aspergillus* spp *sec. Versicolores* detected in the survey and related type- or reference-species strains. The tree was rooted with *A. steynii NRRL 35675*. Numbers at nodes indicate support for the internal branches within the resulting trees obtained by bootstrap analysis.

Table 3
Mycotoxin production *in vitro*.

ISOLATE	SPECIES	OTA	AFB1	AFB2	STC	CIT	PAT
N43	*A. amoenus*	–	–	–	–	n.t.	n.t.
T363	*A. flavus*	–	+	+	n.t.	n.t.	n.t.
L2, K71	*A. jensenii*	–	–	–	–	n.t.	n.t.
M11	*A. tennesseensis*	–	n.t.	n.t.	n.t.	–	–
T18	*A. puulaauensis*	–	+	–	+	n.t.	n.t.
T111, T112, T16, T32, T33,W31	*A. steynii*	+	n.t.	n.t.	n.t.	n.t.	n.t.
A101, A111, A112, A121, A122, A211, A221, A301, A302, A311, A321, B101, B201, B301, B302, K51, K61, M22, N11, N21, N31, N71, N81, N82, N91, O11, O21, P11, T31	*A. westerdijkiae*	+	n.t.	n.t.	n.t.	n.t.	n.t.
M31, M51, L112	*A. westerdijkiae*	–	n.t.	n.t.	n.t.	–	–
C2, C3, C5	*P. biforme*	–	n.t.	n.t.	n.t.	n.t.	n.t.
A303	*P. gravinicasei*	–	–	n.t.	–	–	–

LOD: OTA = 2 μg/kg, AFB1 = 0.2 μg/kg, AFB2 = 0.04 μg/kg, ST = STC = 4 μg/kg, CIT = 5 μg/kg, PAT = 5 μg/kg n.t. = not tested.

its presence is linked to occurrence of ochratoxigenic molds, specifically of *A. westerdijkiae* and *A. steynii*, detected on the surface of some cheeses (around 40%). Only sample "V" resulted positive to OTA (0.8 μg/kg) even though none ochratoxigenic species has been isolated from it. The apparent incongruity could be due to the random sampling of microorganisms on the rind, which is not completely representative of the whole mycobiota, not homogenously distributed on the surface.

On the other hand, we detected potentially ochratoxigenic fungi on sample "L", resulted negative to OTA content, probably due to environmental conditions, commonly narrow than allowing fungal growth, or to chemiotype of fungal isolates, probably OTA not-producing strain (as resulted by *in vitro* tests for L112, M31, and M51).

Table 4
Mycotoxin occurence in cheeses.

Sample ID	AFM1 (μg/kg)	OTA (μg/kg)	AFB1 (μg/kg)	STC (μg/kg)	PAT (μg/kg)
A	–	317,07	–	–	–
B	–	49,08	–	–	–
C	–	–	–	–	–
D	–	–	–	–	–
E	–	–	–	–	–
F	–	–	–	–	–
G	–	–	–	–	–
H	–	–	–	–	–
J	–	–	–	–	–
K	–	–	–	–	–
L	–	–	–	–	–
M	–	0,5	–	–	–
N	–	3,3	–	–	–
O	–	–	–	–	–
P	–	0,2	–	–	–
R	–	–	–	–	–
S	–	–	–	–	–
T	–	3,2	–	–	–
U	–	–	–	–	–
V	–	0,8	–	–	–
W	–	0,2	–	–	–
Z	–	–	–	–	–

– = not detected. LOD: AFM1 = 0.4 μg/kg, OTA = 0.1 μg/kg, AFB1 = 0.1 μg/kg, STC = 0.5 μg/kg, Patulin = 0.5 μg/kg.

Table 5
Mycotoxin occurence in sample "A" and "B".

Cheese subsample	OTA content (μg/kg)	Ochratoxigenic fungal species isolated
A-1-0	317.07	*A. westerdijkiae* (OTA +)
A-1-1	61.55	
A-1-2	2.20	
A-2-0	2812.20	
A-2-1	2.80	
A-2-2	4.68	
A-3-0	6748.45	
A-3-1	2.40	
A-3-2	3.65	
B-1-0	9.31	*A. westerdijkiae* (OTA +)
B-1-1	n.r.	
B-1-2	n.r.	
B-2-0	4.00	
B-2-1	n.r.	
B-2-2	n.r.	
B-3-0	49.08	
B-3-1	n.r.	
B-3-2	n.r.	

OTA LOD: Cheese = 0,02 μg/kg; Fungal species = 1 μg/kg.

Cheeses produced in northern area of Italy did not be settled by fungal toxigenic species and secondary metabolites were absent. The irregular distribution of OTA contamination around rinds of samples "A" and "B" suggests a link between mycotoxin contamination and discontinuous growth of fungus. The measured gradient from rind to core of cheese suggests a deeper investigation about possible OTA migration in cheese, similarly to OTA migration in dry-cured meat (Ferrara et al., 2016), but more worrisome due to the absence of a barrier between mycelia and edible part of product, equivalent to casing in sausages. Actually, in some dairies the cave cheeses are used to be coated by vegetable matrixes (such us mixtures of herb, ash, leaves, spices, oils, pistachios, walnuts, aot) which can either prevent mycotoxins or fungal mycelium diffusion into the cheese, either could carry over potential toxigenic fungal population on rind. Furthermore, the few data collected about cheese ripening time seem to be not related to the extension of period. For example, the sample "A", ripened for 9 months, have in the rind around 700 or 1500 times more OTA levels than samples "M" and "P", ripened for 11 an 18 months, respectively. However, our data demonstrate that cave cheese is a susceptible product to toxigenic fungi growth and also to secondary metabolite contaminations, mostly OTA produced by *A. westerdijkiae* and/or *A. steynii*, which could affect inner part of the cheese, not only the rind.

Intake of OTA from cheese seems to be of limited importance for the general population in comparison to potential OTA intake from cereals and their derived products, and therefore no limit is defined for OTA in cheese. Really, removing rind would greatly reduce the possibility of ingesting mycotoxins, as usually done by Italian consumers eliminating the mold layer before eating. However, consumers have different customs in cheese consumption. Ancient Italian traditions consider rind as mostly edible part and consumption could increase possible mycotoxin risk than in other regions of Italy. Therefore, to ensure continued production of safe and high-quality artisan cave cheese, mycotoxins should be contemplating too, maybe defining a specific limit for OTA content in cave cheese or monitoring presence of undesirable ochratoxigenic species, *A. westerdijkiae* and *A. steynii*. Monitoring those species, widespread in caves and caves-like environments, as well as selection of safe native secondary starters, could be effective solutions to keep out undesirable fungal population, in particular mycotoxigenic fungi, following sausage production protocols (Sunesen and Stahnke, 2003; Bernáldez et al., 2013), and could be incorporated into the hygienic production system in the framework of HACCP. Finally, the "rind edible" claim should not be allowed on tags of uncontrolled artisan cheese.

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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.2018.09.014.

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