# Production and migration of ochratoxin A and citrinin in Comté cheese by an isolate of *Penicillium verrucosum* selected among *Penicillium* spp. mycotoxin producers in YES medium

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ABSTRACT

Moldy food products that are not subject to pathogenic bacterial contamination could be trimmed by consumers to remove fungal mycelium before consumption. However, prior to giving such recommendations to consumers, it is necessary to evaluate potential mycotoxin migration in these products. This study aimed at quantifying citrinin (CIT) and ochratoxin A (OTA) accumulation and migration in a French semi-hard Comté cheese after artificial inoculation with a CIT- and OTA-producing *Penicillium verrucosum* strain. At 8 °C, CIT and OTA production started after 14 days and 28 days incubation, respectively; while at 20 °C, both mycotoxins were produced from day 7. At 20 °C, maximum CIT concentration, about 50000 ng/g, was 20 fold that at 8 °C. Regardless of temperature, maximum OTA concentration was about 4000 ng/g cheese. Maximum concentrations were obtained in the upper part of the cheese, but depending on incubation time, mycotoxins were detected up to 1.6 cm in depth. As long as only white mycelium developed on the cheese surface, trimming can be acceptable, but a blue mold color (due to fungal sporulation) was associated with the accumulation of significant amounts of mycotoxins so the product should be discarded.

## 1. Introduction

In Europe, North America and Oceania, more than 10% of milk and dairy products are lost, principally at the consumer level (FAO, 2011). Microbial spoilage of food products is one of the reasons for these losses. In contrast to bacteria, mold contamination is easily detected by the consumer due to the presence of a noticeable mycelium, but due to insufficient knowledge about molds, spoiled products are usually thrown in the bin. However, some websites provide food safety information. For example, it is recommended to discard mold contaminated jams, yogurt, soft cheese, and bread, and to cut off at least one inch around and below the mold spot on hard cheese because mold cannot penetrate deep into the product (USDA, 2013). Soft cheese should be discarded because moldy foods may also have unwanted bacteria growing along with the mold. In contrast to soft cheeses, semi-hard and hard cheeses have not been reported to be subject to contamination by pathogenic bacteria. For example, it was previously shown that mild, reduced fat and sharp Cheddar did not support growth of *Listeria monocytogenes* and *Salmonella* spp. (Leong et al., 2014).

The main problem with mold spoiled foods is the possible production of mycotoxins that can diffuse from the mycelium to the product. Mold spots cannot simply be scraped off or removed from the product because the amount of mycotoxins at a given place in a given product depends on many factors such as product composition, microorganism, extent of contamination, environmental conditions, and distance to the mycelium (where mycotoxin production actually takes place). Therefore, any recommendation to discard or to cut off part of the product should be supported by quantifying mycotoxin diffusion in the food product. Studies on this topic are scarce. The ability of aflatoxins, ochratoxin A (OTA), and citrinin (CIT), produced by *Aspergillus parasiticus*, *Aspergillus ochraceus*, and *Penicillium chrysogenum*, respectively, to diffuse in bread was assessed by Reiss (1981). OTA formation was high in the first slice (1 cm thickness) and decreased in the subsequent slices whereas CIT production was only detected in the uppermost slice. Patulin (PAT) distribution was also determined in decayed apples, but only for a 20 mm diameter lesion (Bandoh et al., 2009). In another study, the decrease in PAT concentration as a function of the distance from the infected tissue in apples and tomatoes was about 2 log/cm and less than 1 log/cm, respectively (Rychlik and Schieberle, 2001). In sweet peppers, fumonisin B$_1$ was detected 1.5 cm

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away from the mold lesion (Monbaliu et al., 2010). More recently, dairy products, jams and tomato puree were inoculated by *Penicillium expansum*, *Penicillium roqueforti*, *Penicillium crustosum*, *Aspergillus versicolor*, and *Penicillium commune* (Olsen et al., 2017).

Surprisingly, mycotoxin diffusion was never assessed in cheese. Obviously, soft cheeses should not be considered for this kind of study because bacterial growth, especially pathogens, may also occur along with the mold. Therefore, discarding the moldy product is the only safe recommendation for consumers. In contrast to soft cheeses, evaluating mycotoxin diffusion in semi-hard and hard cheeses would provide reliable data to determine the size of the portion around the mold that should be cut-off. Comté is a semi-hard cheese, purchased by more than half of French households, and is by far the first protected designation of origin (PDO) cheese in France with a total annual production of 64,179 tons in 2014 (Eugster-Meier et al., 2018). By comparison, the production of Gruyere and Emmental PDO cheeses in Switzerland were 26,300 and 23,480 tons, respectively in 2010 (Anon, 2018). The minimum ripening time for Comté cheese is 4 months, but this time can be extended up to 48 months. During ripening, water activity varies from 0.962 (2 months) to 0.959 (4 months) (Piton-Malleret and Gorrieri, 1992). A much greater variation in $a_w$ between 4 and 48 months ripening can be expected. For example, in the case of Cheddar cheese, $a_w$ was shown to decrease linearly from 0.965 to 0.956 during the first 270 days of ripening due to an increase of proteolysis (Hickey et al., 2013).

Several investigators have reported that the dominant contaminating fungi isolated from cheese are *Penicillium* spp. (Northolt et al., 1980; Bullerman, 1981; Lund et al., 1995; Barrios et al., 1998; Garnier et al., 2017). Lund et al. (1995) described *P. commune*, *Penicillium palitans*, *Penicillium nalgiovense*, and *Penicillium verrucosum* species as the most frequent contaminants of hard, semi-hard, and soft cheeses. Reports of *P. verrucosum* occurrence in cheese should however be considered with care as many *P. verrucosum* isolates have been reclassified to the closely related *Penicillium nordicum* species (Larsen et al., 2001). *P. nordicum* is well adapted to salt-rich environments and foods, including cheeses, and is often a consistent producer of OTA while *P. verrucosum* is found in other habitats (e.g., cereals) as well as salt-rich foods like cheese and can produce OTA and/or CIT depending on growth and environmental conditions (Giesen et al., 2018). For these species, OTA biosynthesis may be related to strain fitness and more precisely to the capacity to adapt to a salt-rich environment like cheese. For *P. commune*, this species represented 45% of the *Penicillium* isolates in the study by Lund et al. (1995) and is considered as the dominant fungus isolated from cheeses. Most isolates produced cyclopiazonic acid (CPA) (Frisvad and Filtenborg, 1989) but also some other secondary metabolites (i.e., rugulovasines and viridicatin) on cheese agar (Larsen et al., 2002).

In cheese, the most hazardous mycotoxins are aflatoxin M1 (AFM1) and OTA (Hymery et al., 2014). AFM1 is the only mycotoxin for which regulatory maximum levels have been set (0.05 and 0.5 μg/kg in the milk used for cheese-making in the EU [Commission Regulation [EC] Nr. 1881/2006] and United States and China, respectively). However, this mycotoxin is already present in milk and is not produced during cheese storage. Maximum OTA levels are regulated in the EU (Commission Regulation [EC] Nr. 1881/2006) for some foods but not cheese. However, due to its toxicity, OTA was classified by the IARC (1993), as class 2B, possibly carcinogenic to humans. On the basis of the lowest observed adverse effect level (LOAEL) of 8 μg/kg body weight per day for early markers of renal toxicity in pigs (the most sensitive animal species), and applying an uncertainty factor of 450 for the uncertainties in the extrapolation of experimental data derived from animals to humans as well as for intra-species variability, EFSA (2006) established a Tolerable Weekly Intake (TWI) of 120 ng/kg b. w. The majority of *P. verrucosum* isolates are OTA producers and some isolates also produce CIT (Frisvad and Samson, 2004). *Penicillium citrinum* and *P. verrucosum* are the only known *Penicillium* species to produce this mycotoxin in

cheese (Hymery et al., 2014). CIT, like OTA, is nephrotoxic, but in group 3 as it is not classifiable as to its carcinogenicity towards humans (IARC, 1987). Citrinin is nephrotoxic and a no-observed-adverse-effect level (NOAEL) of 20 μg/kg body weight per day was identified from a 90-day study in rats. Due to the limitations and uncertainties in the database, the derivation of a health-based guidance value was not considered appropriate but a level of no concern for nephrotoxicity of 0.2 μg/kg b. w. per day was determined. Based on the available data a concern for genotoxicity and carcinogenicity could not be excluded at the level of no concern for nephrotoxicity, (EFSA, 2012). Based on toxicity to animals, other *Penicillium* mycotoxins such as penitrem A, a powerful neurotoxin produced by *Penicillium crustosum*, could also be tested for migration in cheese.

Various isolates of *Penicillium* spp. were first screened for their ability to produce mycotoxins on Yeast Extract Sucrose (YES) Agar and their ability to grow and produce mycotoxins on Comté cheese. Then, the selected mycotoxin producing isolate was grown on Comté cheese at both refrigerated, 8 °C, and ambient, 20 °C, temperatures. In order to determine the quantity of cheese that should be cut-off before consumption, mycotoxins were quantified in 2 mm thick slices depthwise and mycotoxin concentrations were correlated to the visual aspect of the moldy cheese.

## 2. Materials and methods

### 2.1. Fungal isolates

All *Penicillia* (belonging to *P. crustosum*, *P. nordicum* and *P. verrucosum*) were isolated from cheese or dairy related environments, with the exception of *P. verrucosum* UBOCC-A-109221, and belong to either the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France) or were part of the LUBEM laboratory culture collection (Table 1). The origin of *P. verrucosum* UBOCC-A-117113 was unknown. These isolates were selected as they belong to the main known *Penicillium* species that can contaminate semi-hard cheeses. All species were previously identified in the laboratory according to partial β-tubulin gene sequencing as described by Ropars et al. (2012). Stock cultures were maintained at −80 °C.

### 2.2. Media

For conidial production, Potato Dextrose Agar (PDA, BD Difco, France) was used and plates were incubated at 25 °C for 7 d after inoculation. For mycotoxin determination, Yeast Extract Sucrose (YES) agar medium (Frisvad and Filtenborg, 1989) buffered at pH 4.5 with phosphate-citrate buffer (Chang et al., 1998) was used as described by Gillot et al. (2017), as this medium is characterized by a high C/N ratio favoring mycotoxin production (Visagie et al., 2014).

**Table 1**
List of *Penicillium* isolates tested for their ability to produce mycotoxins in cheese.

| Species | Isolate code | Origin | Country | Year |
|---|---|---|---|---|
| *Penicillium crustosum* | 16057 | Brie cheese | France | unknown |
| | 16054 | Spreadable cheese | France | unknown |
| | 12051 | Yogurt drink | France | unknown |
| *Penicillium nordicum* | 5i9 | Raclette | France | 2012 |
| *Penicillium verrucosum* | UBOCC-A-117113 | Unknown | Belgium | before 2016 |
| | UBOCC-A-111269 | Aromatized dairy drink | France | 2003 |
| | UBOCC-A-109221 | Syrup | France | 2009 |

## 2.3. Cheese

The French Comté cheese used throughout this study was produced by the Fromagerie Vagne (Poligny, France). All samples were taken from the same circular cheese wheel (batch number FR39.434.002CE). Comté is a semi-hard cheese, ripened for 6 months, with a water activity of 0.95.

## 2.4. Conidia production

Fungal stock cultures (1 mL) were thawed at ambient temperature for about 1 h. Plates were then inoculated with 0.1 mL of the stock culture (ca. $1 \times 10^6$ conidia. $mL^{-1}$) and incubated at 25 °C for 7 d. Fresh conidia were harvested using a sterile loop after flooding the surface of the plates with 2 mL of distilled water containing 0.01% Tween 80 (Sigma Aldrich, Saint Quentin Fallavier, France). After counting conidia using a haemocytometer, conidial suspensions were standardized to $1 \times 10^6$ conidia. $mL^{-1}$. Suspensions were used directly after preparation for the different experiments.

## 2.5. Isolate screening for mycotoxin production in YES medium

For mycotoxin determination, 1 µL of each spore suspension ($10^6$ conidia. $mL^{-1}$) were inoculated in the center of YES plates and incubated for 7 days under optimal growth conditions at 25 °C. Each culture was performed in triplicate. After incubation, the content of the agar plates was stored at −20 °C prior to mycotoxin analysis.

In parallel, fungal growth was also monitored and the corresponding dry weights were correlated to the obtained mycotoxin levels determined by QTOF LC-MS in YES media. For dry weight determinations, 1 µL of each spore suspension ($10^6$ conidia. $mL^{-1}$) were inoculated in the center of YES plates containing sterile cellophane disks (Carrefour, France). Plates were incubated for 7 days at 25 °C. After incubation, cellophane disks containing fungal mycelium were carefully removed from the plates and placed into glass Petri dishes. Fungal mycelia were removed from the cellophane disks by adding sterile distilled water. Dry weights were then determined after drying mycelia at 70 °C for 48 h. Each culture was performed in triplicate.

## 2.6. Isolate screening for mycotoxin production in Comté cheese

Cheese rind was discarded aseptically. Cheese was then cut into $4 \times 4 \times 0.2 \, cm^3$ pieces and placed in Petri dishes. Then, 30 µL of a $10^6$ conidia. $mL^{-1}$ suspension were spread over the surface with a sterile bent glass rod. Petri dishes were placed on a pedestal in hermetically closed plastic containers. To control cheese water activity, a 20% glycerol (w/w) aqueous solution was poured into the bottom of containers. Triplicates were incubated for 10 d at 20 °C and each cheese piece was stored at −20 °C prior to mycotoxin analysis.

## 2.7. Experimental conditions for mycotoxin migration in cheese

The procedure was identical to §2.6 except that cheeses were cut into $4 \times 4 \times 3 \, cm^3$ cubes before inoculation (or not for control cheeses) with 30 µl of $10^6$ conidia. $mL^{-1}$. Spore suspensions were evenly spread

on each cheese surface before being placed in sterile glass jars. Jars were then placed in larger hermetically closed containers with controlled water activity using the same solution as in §2.6. Triplicates were incubated for 42 d at 8 °C and 28 d at 20 °C.

For each sampling date (0, 7, 14, 21, 28, 35 and 42 d), photographs of fungal growth on the cheese surface were taken and mycotoxin analyses in increasing depthwise cheese fractions were performed in triplicate. To do so, 2 mm thick cheese slices were cut from the cheese cube depthwise and named D0 (moldy upper slice from 0 to 2 mm), D1 (trimmed upper slice from 0 to 2 mm without mold, obtained from an identical replicate), then the other slices obtained from the first cube were numbered D2 (2–4 mm) to D8 (14–16 mm). All cheese slices were stored at −20 °C prior to mycotoxin analysis.

## 2.8. Non-selective extraction of fungal metabolites from YES medium

For YES media, cultures (mycelium and medium) were first homogenized with an Ultraturrax T25 digital (IKA, Heidelberg, Germany) for 1 min. Then, a 4 g aliquot was added to 25 mL of acetonitrile (ACN) containing 0.1% formic acid (v/v), vortexed for 30 s, then sonicated for 15 min. Samples were again vortexed for 30 s before centrifugation at 5000g for 10 min at 4 °C. Ten mL of the ACN phase were collected and stored at −20 °C until QTOF LC-MS analysis. Before analysis, 1 mL was filtered through a 0.2 µm PTFE membrane syringe 4 mm filter (Phenomenex, Torrance, USA) into an amber vial.

## 2.9. Non-selective extraction of mycotoxins from cheese samples

Cheese samples were aseptically cut into small pieces using a scalpel, placed in 15 mL tubes, then 6.25 mL of diluted ACN (84%) supplemented with 2.5% acetic acid (v/v) per g of cheese were added. Samples were vortexed for 2 min, homogenized with an Ultraturrax T25 digital (IKA, Heidelberg, Germany) for 1 min, then 10 mL hexane were added. Samples were placed on a RotoFlex Plus tube rotator (Sigma, France) for 30 min at room temperature, sonicated for 15 min and centrifuged at 4000 g for 10 min at 4 °C. After discarding the hexane layer, 5–10 mL of the ACN phase were collected and stored at −20 °C until QTOF LC-MS analysis. Before analysis, 1 mL of the solution was filtered through a 0.45 µm PTFE membrane syringe 4 mm filter (Phenomenex, Torrance, USA) into an amber vial.

## 2.10. Fungal metabolite detection and quantification by QTOF LC-MS

Extracted fungal extrolites, including mycotoxins, were detected and quantified using an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (QTOF) LC-MS system (Agilent Technologies, Santa Clara, CA, USA). The HPLC system included a Binary pump 1260 and degasser, well plate autosampler set to 10 °C and a thermostated column compartment.

Mycotoxin standards used for quantification were: citrinin (CIT), ochratoxin A (OTA), penitrem A (PEN A) and roquefortine C (ROQ C). Standards were obtained from Sigma-Aldrich (St Louis, MO, USA) except for PEN A from Cfm Oskar Tropitzsch (Marktredwitz, Germany). All mycotoxin stock solutions were prepared in dimethyl sulfoxide (DMSO) at $1 \, mg \, mL^{-1}$ and stored at −20 °C in amber vials. Calibration

**Table 2**
Target mycotoxin quantification parameters used for QTOF LC-MS analyses. RT: retention time (*RT values obtained for isolate screening), MW: molecular weight, ESI: electrospray ionization, DL: detection limit, QL: quantification limit.

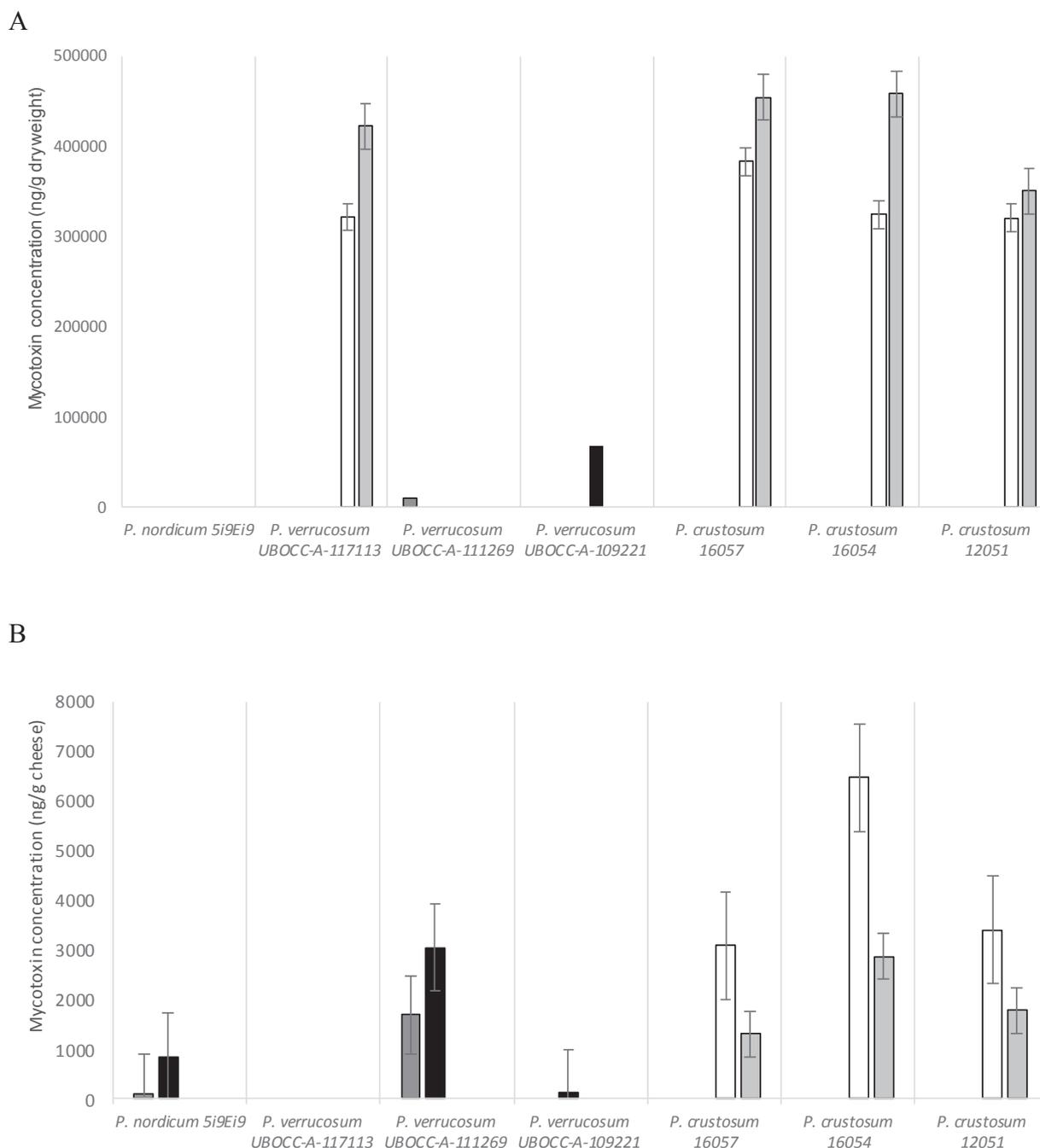| Metabolite | Chemical formula | RT (min) | MW (g/mol) | Quantifier ion (m/z) | Qualifier ion (m/z) | ESI mode | DL (ng/g) | QL (ng/g) | $R^2$ |
|---|---|---|---|---|---|---|---|---|---|
| Citrinin | $C_{13}H_{14}O_5$ | 12.1 | 250.2 | 251.0 | 273.0 | + | 19.2 | 58.2 | 0.99 |
| Ochratoxin A | $C_{20}H_{18}ClNO_6$ | 14.8 | 403.8 | 404.0 | 426.0 | + | 20.5 | 61.7 | 0.99 |
| Penitrem A | $C_{37}H_{44}ClNO_6$ | 29.1* | 634.2 | 632.2 | 668.2 | - | 199.0 | 602.7 | 0.99 |
| Roquefortine C | $C_{22}H_{23}N_5O_2$ | 15.1* | 389.5 | 390.1 | na | + | 136.8 | 414.5 | 0.98 |

A



B



**Fig. 1.** Specific mycotoxin production by seven *Penicillium* species isolates after 7 d growth at 25 °C on YES medium (A) or 10 d growth at 20 °C on 6 month ripened Comté cheese (B). Quantified mycotoxins: CIT (dark grey), OTA (black), PEN A (white) and ROQ C (light grey).

curves prepared in ACN ranged from 10 to 5000 ng mL$^{-1}$. All compound characteristics for mycotoxin identification and quantification are provided in Table 2.

For screening isolates for mycotoxin production (YES medium or cheese samples), 2 μL filtered samples were injected and separated using a ZORBAX Extend-C18 column (2.1 × 50 mm and 1.8 μm, 600 bar). The column was maintained at 35 °C and the flow rate was set to 0.3 mL min$^{-1}$ using as mobile phase: solvent A (milli-Q water + 0.1% LC-MS formic acid v/v (Carlo Erba Reagents, France) + 0.1% ammonium formate v/v (Thermo Fisher Scientific, Waltham, MA, USA)) and solvent B (100% LC-MS grade ACN). Solvent B was maintained at 10% for the first 3 min, followed by a gradient of 10–100% of B for 42 min. Finally, solvent B was maintained at 100% for 5-min post-time. Analytes were ionized in both ESI+ and ESI-

modes (ESI: Electrospray Ionization). Mass spectrometer conditions were: capillary voltage, 4.0 kV; source temperature, 325 °C; nebulizer pressure, 50 psig; drying gas, 12 L/min and ion range, 100–1000 m/z. Mycotoxin detection and identification was performed using retention time values ± 1 min and the corresponding quantifier or qualifier ions for each mycotoxin standard listed in Table 2. Mycotoxin concentrations were calculated from the equation y = mx + b, as determined by weighted (1/x$^2$) linear regression of the matrix-matched calibration data. For YES samples, these values were also correlated to the calculated fungal biomass (determined dry weight values) and specific mycotoxin production was expressed as ng per g dry weight. In contrast to YES medium, mycotoxin production was expressed as ng per g cheese for Comté cheese.

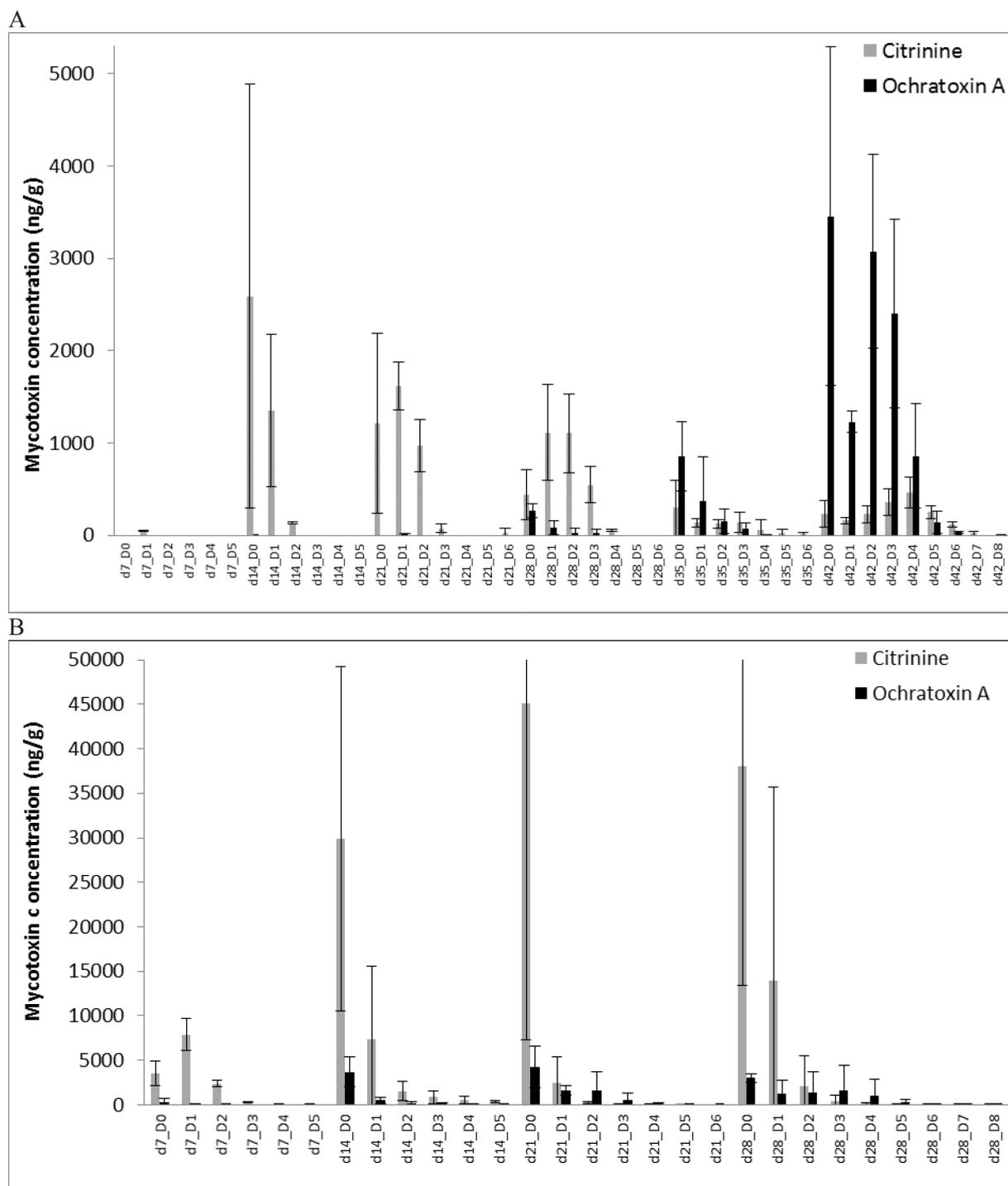For mycotoxin migration experiments in Comté cheese, some

**Fig. 2.** CIT (dark grey) and OTA (black) quantification in Comté cheese, inoculated with *P. verrucosum* UBOCC-A-111269, after 42 and 28 days incubation at 8 °C (A) and 20 °C (B), respectively. d: day; D0: 0–2 mm depth with mould, D1: 0–2 mm depth without mould, D2: 2–4 mm depth, D3: 4–6 mm depth, D4: 6–8 mm depth, D5: 8–10 mm depth, D6: 10–12 mm depth, D7: 12–14 mm depth, D8: 14–16 mm depth.

changes were made to optimize the quantification of the two target mycotoxins CIT and OTA as follows: 2 µL filtered samples were injected and separated in a Poroshell 120 EC-C18 column (2.1 × 50 mm and 2.7 µm, 600 bar) maintained at 35 °C. The flow rate was set to 0.3 mL min$^{-1}$ using as mobile phase: solvent A (milli-Q water + 0.1% LC-MS formic acid v/v + 0.1 M ammonium formate (Carlo Erba Reagents, France)) and solvent B (100% LC-MS grade CAN + 0.1% LC-MS formic acid v/v (Carlo Erba Reagents, France)). Solvent B was maintained at 10% for the first 4 min, followed by a gradient of 10–100% of B for 16 min. Finally, solvent B was maintained at 100%

2 min followed by 5 min post-time. Target analytes were ionized in ESI + mode and identical mass spectrometer conditions were used as described above. Mycotoxin detection and identification was performed as above and specific mycotoxin production was expressed as ng per g cheese.

Method performance and validation parameters including linearity (R$^2$), detection limit (DL), quantification limit (QL), recovery values and matrix effects were also assessed for target metabolites of interest according to the ICH guidelines (ICH Harmonized Tripartite Guideline, 2005). The calibration method used for reliable metabolite
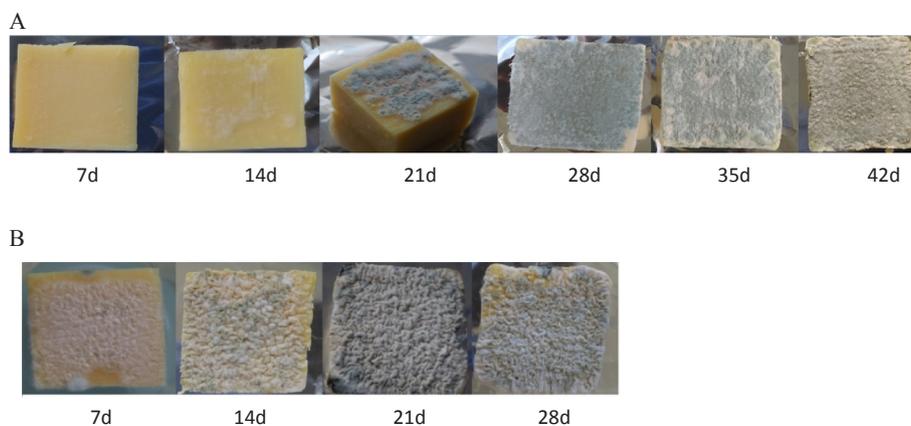
A

B



**Fig. 3.** Weekly monitoring of fungal growth on semi-hard Comté cheese during incubation at 8 °C (A) and 20 °C (B).

quantifications was prepared as previously described (Fontaine et al., 2015). Briefly, blank matrix (Comté cheese) aliquots were treated using the sample preparation procedure described above to obtain blank extracts. Each blank extract was then spiked with an adequate volume of the stock solutions of the two target mycotoxins, CIT and OTA, to reach concentrations ranging from 5 to 5000 ng mL$^{-1}$. Calibration curves were obtained by plotting the peak area against mycotoxin concentrations using a $1/x^2$ weighted linear regression model. According to ICH guidelines (ICH Harmonized Tripartite Guideline, 2005), DL and QL of each metabolite was obtained by multiplying the standard deviation of y-intercepts of regression lines divided by the slope, by 3.3 and 10, respectively (Table 2). Recovery percentage was evaluated in triplicate using cheese matrices spiked with a mix of CIT and OTA at 250 and 2500 ng g$^{-1}$ (recovery > 85% for both concentrations). Finally, all calibration curve calculations were carried out using the Agilent MassHunter Workstation Software (Agilent Technologies, Santa Clara, CA, USA).

## 3. Results

### 3.1. Screening of Penicillium isolates for mycotoxin production

A total of seven *Penicillium* isolates belonging to 3 species already described in the literature as mycotoxin producers in dairy products were selected, namely *P. nordicum* (isolate 5i9), *P. verrucosum* (isolates UBOCC-A-117113, UBOCC-A-111269 and UBOCC-A-109221) and *P. crustosum* (isolates 16057, 16054 and 12051), to determine their mycotoxin production profiles in both YES medium and Comté cheese.

With the notable exception of *P. nordicum* (< DL for all target metabolites in YES agar), isolates belonging to the other two species produced mycotoxins in YES medium after 7 d incubation at 25 °C (Fig. 1a). Major mycotoxins produced by all 3 *P. crustosum* isolates and by one *P. verrucosum* isolate (UBOCC-A-117113) were penitrem A (PEN A) and roquefortine C (ROQ C). *P. verrucosum* UBOCC-A-109221 was the only isolate that produced quantifiable amounts of OTA on YES medium. As observed for the YES substrate, all *P. crustosum* isolates also produced PEN A and ROQ C in Comté cheese (Fig. 1a). *P. nordicum* produced OTA and CIT in Comté cheese while these metabolites were not detected in YES agar extracts. In contrast to YES medium, *P. verrucosum* UBOCC-A-117113 and UBOCC-A-109221 did not produce any mycotoxins in Comté cheese (Fig. 1b). *P. verrucosum* UBOCC-A-111269 was the only isolate to simultaneously produce significant levels of OTA and CIT in Comté cheese (Fig. 1b). Therefore, this isolate was selected to determine the mycotoxin migration in a cheese substrate.

### 3.2. Migration of mycotoxins in Comté cheese

To mimic mold contamination that occurs at the consumer level on unpackaged cheese by sources such as the knife used for cutting, the cheese wheel, ambient air or the fridge, *P. verrucosum* was artificially inoculated on the surface of a rind-free Comté cheese then incubated at controlled temperature (8 °C refrigerated temperature and 20 °C ambient temperature) and water activity conditions for up to 42 days.

At 8 °C, production of CIT and OTA started after 14 and 28 days incubation, respectively (Fig. 2a). At 14 days, CIT was mainly detected in 2 mm depth samples with and without fungal mycelium although highest concentrations where detected when the mold was present. Low CIT concentrations (about 125 ng/g) were also detected from 2 to 4 mm in depth. At 21 days, significant amounts of CIT, about 1500 ng/g and 1000 ng/g, were detected in the 0–2 mm and 2–4 mm slices, respectively, as well as very low levels between 4 and 6 mm (< 75 ng/g). At 28 days, CIT was detected at concentrations ranging from 1100 to 500 ng/g up to 6 mm depth, as well as at about 50 ng/g up to 8 mm depth, but the concentrations at the cheese surface decreased, thus suggesting that the production of CIT at the surface of the cheese had stopped and consequently that CIT had migrated into the depth of the cheese. At 28 and 35 days, OTA was produced at concentrations reaching about 250 ng/g and 850 ng/g, respectively, and detected up to 6 mm in depth by 35 days. At both 35 and 42 days, OTA production was higher than CIT and by 42 days, OTA concentrations reached maximum levels with up to about 3500 ng/g cheese. At this time, OTA was detected at a depth greater than 10 mm. Mycotoxin production was then correlated to changes in fungal growth after incubation at 8 °C (Fig. 3a). Up to 14 days, loose white mycelium was observed on the surface of cheeses incubated at 8 °C (Fig. 3a). By 21 days, a blue color due to the production of conidia appeared. Then, from 28 to 42 days, blue mold completely covered the cheese surface which corresponded to the same time period when OTA was produced.

At 20 °C, production of both mycotoxins began at day 7 and CIT levels were systematically higher than OTA in the 0–2 mm slices (Fig. 2b). At 20 °C, the maximum CIT concentration, about 50000 ng/g, was 20 fold that at 8 °C. The highest CIT concentrations were obtained in the upper part, 0–2 mm, of cheese for all sample dates. In contrast to 8 °C, CIT concentrations did not decrease significantly from 14 to 28 days. At 28 days, about 80 ng/g were quantified at 6–8 mm in depth, then concentrations decreased to levels < 50 ng/g in the remaining tested slices. In contrast to CIT, maximum OTA concentrations at 20 °C (~4200 ng/g) did not differ significantly from those produced at 8 °C. At 21 and 28 days, OTA was detected at concentrations > 50 ng/g at depths up to 6–8 mm and 8–10 mm, respectively. As for CIT, OTA concentrations then decreased to levels < 50 ng/g in the remaining analyzed samples.

Fungal growth at 20 °C was characterized by white mycelium largely covering the cheese surface after 7 days incubation (Fig. 3b). Blue mold aspect due to conidia production was observed from day 14 only at 20 °C versus day 21 for 8 °C (Fig. 3a). A dense blue mold layer was

then visible at 21 d and 28 d incubation at 20 °C. Highest OTA and CIT quantities were again produced when blue mold was visible on the cheese surface.

## 4. Discussion

Mycotoxin production profiles were first determined by QTOF LC-MC for 7 isolates belonging to 3 known mycotoxin producing *Penicillium* species - *P. nordicum*, *P. verrucosum* and *P. crustosum* - that can contaminate dairy foods using two different substrates, YES agar and Comté cheese. Our results showed that, in some cases, different mycotoxins were produced depending on the growth substrate used to cultivate the fungal isolates. This observation has already been described for the same 3 *Penicillium* species using 3 substrates by Kokkonen et al. (2005). ROQ C and PEN A were synthesized on both substrates by all 3 *P. crustosum* isolates. These species have already been described to produce these compounds in different substrates including foods (Kokkonen et al., 2005; Leggierei et al., 2017; Olsen et al., 2017; Prencipe et al., 2018). ROQ C and PEN A were produced by *P. verrucosum* UBOCC-A-117113 on YES agar but not in cheese. It was reported that this species was able to produce ROQ C, (Kozlovsky and Reshetilova, 1984), but this result was later suggested to be incorrect by Frisvad et al. (2004). *Penicillium verrucosum* var. *cyclopium* RV 67718, isolated from ground cassava collected in Burundi, was cultivated under laboratory conditions to produce the mycotoxin roquefortine C (Musuko et al., 1994). But the production of PEN A by *P. verrucosum* was never reported before. *P. verrucosum* UBOCC-A-117113 and UBOCC-A-111269, identified by the β-tubulin gene, were closely related and clearly identified as *P. verrucosum*. In our opinion, this result was not due to a misidentification, but it should be confirmed by other research groups.

OTA and CIT synthesis were highly variable according to substrate and fungal isolate. *P. nordicum* synthesized OTA in cheese but not in YES medium. This mycotoxin has already been shown to be synthesized at variable concentrations by this species on YES, cheese and bread analogues (Kokkonen et al., 2005) and also according to NaCl content of the substrate (Schmidt-Heydt et al., 2012). The amount of CIT produced by *P. nordicum* in YES medium was below the DL. Finally, 2 *P. verrucosum* isolates were the strongest OTA producers in cheese and 1 isolate also simultaneously produced CIT on this substrate while extracts from YES medium exclusively contained only 1 mycotoxin at a time. The OTA + CIT producing *P. verrucosum* isolate was therefore further studied in our model cheese system to evaluate the potential migration of these compounds into the cheese. Noteworthy, our results highlighted the importance to correctly choose a substrate to determine mycotoxin production profiles of a given strain. We highly recommend testing more than one substrate to determine whether a fungal strain is able to produce one or multiple mycotoxins.

*P. verrucosum* grows from 0 to 31 °C, with an optimum at 20 °C (Pitt and Hocking, 2009). Accordingly, it was observed in our study that the development of this species on Comté was faster at 20 °C than at 8 °C. The increase of temperature from 15 to 20 °C with respect to the same incubation time resulted in an increase in OTA biosynthesis by a factor of 10 (Battilani et al., 2010). In contrast to that study, the concentration of OTA produced in Comté at 8 and 20 °C were not significantly different. The ability to biosynthesize OTA and CIT at 10 °C was not detected on solid cereal substrates (Wawrzyniak and Waśkiewicz, 2014), whereas production of OTA and CIT by *P. verrucosum* was reported on YES medium at 6 °C (Takahashi and Yazaki, 2007). *P. verrucosum* grew and produced CIT at 20 °C and 25 °C, but not at 5 °C, 30 °C, or 35 °C on MEA, CYA, and YES agar (Heperkan et al., 2009). CIT biosynthesis in *P. verrucosum* was shown to be highly regulated by environmental factors like temperature, water activity or pH (Touhami et al., 2018). A higher level of CIT *versus* OTA was produced by *P. verrucosum* at 6 °C on YES medium (Takahashi and Yazaki, 2007). Recent publications suggested that the concentration of CIT may be high in cereals and may even

exceed that of OTA (Vrabcheva et al., 2000; Meister, 2004; Zaied et al., 2012; Kononkenko and Burkin, 2013; Wawrzyniak and Waśkiewicz, 2014). On Comté cheese, maximum amounts of OTA and CIT at 8 °C were of the same order of magnitude although produced at different times, whereas the amounts of CIT were about 10 fold that of OTA at 20 °C. The latter observation strengthened this assumption.

In the present study, the maximum OTA concentration found in Comté cheese was about 3 mg/kg. This amount may depend on the isolate, but also on the substrate. For example OTA has been found in spoiled cheeses, at up to 7 mg/kg (Jarvis, 1983), in moldy cheesecakes, at up to 1 mg/kg (Piskorska-Pliszcynska and Borkowska-Opacka, 1984) and in traditional raw cow milk hand-made semi-hard cheeses at levels ranging from 18 to 146 μg/kg in the cheese interior and 1–262 μg/kg in the rind (Pattono et al., 2013). Up to 600 mg/kg of CIT was also produced on fresh goat cheese by *P. citrinum* after 10 days incubation at 20 °C (Bailly et al., 2002). In the present study, 2.5 mg/kg and 45 mg/kg of CIT were detected in Comté at 8 and 20 °C, respectively, although in this cheese, CIT production was not due to *P. citrinum* but *P. verrucosum*.

*P. nordicum* is adapted to NaCl-rich environments like hypersaline waters (Butinar et al., 2011) or NaCl-rich food products like cheeses or dry-cured meats (Larsen et al., 2001). Important habitats of *P. verrucosum* are cereals and cereal products; however, this species can also occur on salt-rich foods, like dry-cured meats and cheeses, and is obviously responsible for the co-occurrence of OTA and CIT in these types of products (Markov et al., 2013). As expected, the *P. nordicum* isolate tested in our study developed well and produced OTA in Comté, but did not produce any mycotoxins in YES medium. It was reported that strains of *P. verrucosum* exclusively produced CIT on YES, whereas both OTA and CIT were detected in considerable amounts on a bread analogue (Kokkonen et al., 2005). Similarly, the *P. verrucosum* isolate selected in our study for its ability to produce OTA and CIT on Comté cheese, only produced CIT on YES medium.

In *P. verrucosum,* CIT and OTA biosynthesis are mutually regulated (Schmidt-Heidt et al., 2011). Both mycotoxins are polyketides with a highly similar dihydrocoumarin moiety, but in the case of OTA, this moiety contains a chlorine atom and is coupled to the amino acid phenylalanine. High amounts of one of these secondary metabolites are produced at the expense of the other and this species can likely adapt its biosynthesis depending on the encountered environmental conditions in a given habitat. NaCl concentration plays an important role in OTA biosynthesis which can certainly be related to the chlorine atom in its chemical structure. It was suggested that OTA biosynthesis helps ensure chloride homeostasis in the cell in adaptation to a NaCl-rich environment (Schmidt-Heidt et al., 2012). Shift from CIT towards OTA biosynthesis was also reported at increasing NaCl concentrations (Schmidt-Heidt et al., 2012; Giesen et al., 2017). High NaCl concentrations could cause increased intracellular osmotic stress due to intensive mitochondrial respiration (Petrovic, 2006), therefore the antioxidant properties of CIT may also impact adaptation to such an environment (Giesen et al., 2018). At 8 °C, the shift from CIT towards OTA could not be explained by an increasing NaCl concentration, due for example to drying of the surface of the cheese, because water activity in the incubation environment of Comté cheeses was controlled. A change in the pH in Comté cheese, due for example to lipid or protein degradation by the fungus, could explain the shift from CIT to OTA at 8 °C. Also, protein degradation could provide free amino acids, including phenylalanine, in the environment required for OTA biosynthesis. The absence of this shift at 20 °C on Comté cheese could be explained by faster degradation or modification of environmental factors at the surface of Comté, especially as fungal development was very intense on the cheese surface under these conditions.

No study reported that CIT could be an OTA precursor. It rather appears that these two mycotoxins are mutually regulated. Therefore, the decrease in CIT observed after 14 days incubation at 8 °C was not due to a transformation into OTA. The decrease in CIT could rather be explained, at least partially, by the diffusion of this mycotoxin into the

cheese. In fact, by considering the first 4 mm at 21 days incubation, the amount of CIT was about 5000 ng (the weight of each 2 mm slice was about 2 g), as compared to 800 ng at 35 days incubation. By comparison, CIT produced by *P. citrinum* on black olives was maximum at 40 days incubation at 20, 25, and 30 °C than decreased until 60 days (Heperkan et al., 2009). In another study on cheese, it was suggested that the disappearance of this toxin was due to a chemical reaction with cheese components and not to any microbial action (Bailly et al., 2002). Since cheeses are high in casein, it is possible that reactive groups in the protein react with the mycotoxin, causing its disappearance. Previous data have also demonstrated that CIT may be inactivated by cysteine and can be bound to albumin and plasma proteins (Scott, 1977).

At 8 °C, high CIT production and at 20 °C high CIT and OTA production was correlated with a blue colored mold appearance due to the production of conidia. The blue color can be observed by the consumer well after it appears, as storage time may be unknown. Therefore, it is suggested to discard any "blue molded" Comté cheese because mycotoxins were detected up to 16 mm depth in the present study. However, when white mycelium is only detected by the consumer, our results demonstrated that CIT and OTA remained in the first 2 mm of the cheese, therefore trimming (i.e., discarding the upper 2 mm of moldy cheese) can be acceptable.

## 5. Conclusions

In the farm to fork approach, the consumer is an important element of the chain. Regulations can be enforced at the different steps from production to retail, but recommendations can only be suggested to the consumer. With respect to moldy food products, these recommendations are scarce, not easily available and not supported by many scientific experiments. In order to ensure food safety, it should be recommended to discard sell by date products stored in the fridge because mold development can hide a contamination by pathogenic bacteria. Therefore, this study focused on Comté cheese that has not been reported to be contaminated by such bacteria during storage. It should be underlined that the conclusions of the present study should not be extrapolated to other cheeses, especially soft ones. The approach described in the present study was also based on insufficient knowledge of the consumer on mold contamination, i.e., the consumer cannot distinguish a harmless fungi (e.g., Mucoromycota) from potentially toxigenic species. It should also be bared in mind that many people are unaware of mycotoxins, and that mycotoxins can migrate in food products.

The worst case scenario was also privileged. For example, this study was based on the only *Penicillium* isolate capable of producing multiple mycotoxins in Comté cheese. In general, these kinds of studies should select the highest mycotoxin producer, along with the most toxic mycotoxins for humans. Accordingly, OTA and CIT were selected due to their possible genetoxicity and carcinogenicity. Maximum OTA concentrations have been set by the EC 1881/2005 regulation for cereals but not for cheese. In the present study, a great increase in mycotoxin concentration was observed once produced and linked to a blue mold visual aspect. Therefore, it is suggested to discard any part of cheese that would contain detectable mycotoxins.

Considering mycotoxins as chemical hazards is consistent with the objective of setting international regulatory standards based on toxicological studies (Dantigny and Bensoussan, 2013). However, this approach was not suitable to improve food safety at the consumer level simply because mycotoxins cannot be quantified by the consumer. Simple recommendations should be based on the visual aspect of cheese regardless of storage time because many consumers do not know how long food products are stored. Due to slower growth and lower mycotoxin productions at 8 °C than at 20 °C, it can also be recommended to the consumer to store Comté cheese at refrigerated temperatures.

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