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

4

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18

19 **Running title:** Mycotoxin migration in molded cheese

20

21 **Abstract**

22 Moldy food products that are not subject to pathogenic bacterial contamination could be
23 trimmed by consumers to remove fungal mycelium before consumption. However, prior to
24 giving such recommendations to consumers, it is necessary to evaluate potential mycotoxin
25 migration in these products. This study aimed at quantifying citrinin (CIT) and ochratoxin A
26 (OTA) accumulation and migration in a French semi-hard Comté cheese after artificial
27 inoculation with a CIT- and OTA-producing *Penicillium verrucosum* strain. At 8°C, CIT and
28 OTA production started after 14 days and 28 days incubation, respectively; while at 20°C,
29 both mycotoxins were produced from day 7. At 20°C, maximum CIT concentration, about
30 50000 ng/g, was 20 fold that at 8°C. Regardless of temperature, maximum OTA concentration
31 was about 4000 ng/g cheese. Maximum concentrations were obtained in the upper part of the
32 cheese, but depending on incubation time, mycotoxins were detected up to 1.6 cm in depth.
33 As long as only white mycelium developed on the cheese surface, trimming can be
34 acceptable, but a blue mold color (due to fungal sporulation) was associated with the
35 accumulation of significant amounts of mycotoxins so the product should be discarded.

36

37 **Keywords:** food safety, mycotoxins, migration, *Penicillium*, semi-hard cheese

38

39 **1. Introduction**

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

53 The main problem with mold spoiled foods is the possible production of mycotoxins
54 that can diffuse from the mycelium to the product. Mold spots cannot simply be scraped off or
55 removed from the product because the amount of mycotoxins at a given place in a given
56 product depends on many factors such as product composition, microorganism, extent of
57 contamination, environmental conditions, and distance to the mycelium (where mycotoxin
58 production actually takes place). Therefore, any recommendation to discard or to cut off part
59 of the product should be supported by quantifying mycotoxin diffusion in the food product.
60 Studies on this topic are scarce. The ability of aflatoxins, ochratoxin A (OTA), and citrinin
61 (CIT), produced by *Aspergillus parasiticus*, *Aspergillus ochraceus*, and *Penicillium*
62 *chrysogenum*, respectively, to diffuse in bread was assessed by Reiss, (1981). OTA formation
63 was high in the first slice (1 cm thickness) and decreased in the subsequent slices whereas

64 CIT production was only detected in the uppermost slice. Patulin (PAT) distribution was also
65 determined in decayed apples, but only for a 20 mm diameter lesion (Bandoh et al. 2009). In
66 another study, the decrease in PAT concentration as a function of the distance from the
67 infected tissue in apples and tomatoes was about 2 log/cm and less than 1 log/cm, respectively
68 (Rychlik and Schieberle, 2001). In sweet peppers, fumonisin B₁ was detected 1.5 cm away
69 from the mold lesion (Monbaliu et al. 2010). More recently, dairy products, jams and tomato
70 puree were inoculated by *Penicillium expansum*, *Penicillium roqueforti*, *Penicillium*
71 *crustosum*, *Aspergillus versicolor*, and *Penicillium commune* (Olsen et al. 2017).

72 Surprisingly, mycotoxin diffusion was never assessed in cheese. Obviously, soft
73 cheeses should not be considered for this kind of study because bacterial growth, especially
74 pathogens, may also occur along with the mold. Therefore, discarding the moldy product is
75 the only safe recommendation for consumers. In contrast to soft cheeses, evaluating
76 mycotoxin diffusion in semi-hard and hard cheeses would provide reliable data to determine
77 the size of the portion around the mold that should be cut-off. Comté is a semi-hard cheese,
78 purchased by more than half of French households, and is by far the first protected
79 designation of origin (PDO) cheese in France with a total annual production of 64,179 tons in
80 2014 (Eugster-Meier et al. 2018). By comparison, the production of Gruyere and Emmental
81 PDO cheeses in Switzerland were 26,300 and 23,480 tons, respectively in 2010 (Anon, 2018).
82 The minimum ripening time for Comté cheese is 4 months, but this time can be extended up
83 to 48 months. During ripening, water activity varies from 0.962 (2 months) to 0.959 (4
84 months) (Piton-Malleret and Gorrieri, 1992). A much greater variation in a_w between 4 and 48
85 months ripening can be expected. For example, in the case of Cheddar cheese, a_w was shown
86 to decrease linearly from 0.965 to 0.956 during the first 270 days of ripening due to an
87 increase of proteolysis (Hickey et al. 2013).

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

105 In cheese, the most hazardous mycotoxins are aflatoxin M1 (AFM1) and OTA
106 (Hymery et al. 2014). AFM1 is the only mycotoxin for which regulatory maximum levels
107 have been set (0.05 and 0.5 µg/kg in the milk used for cheese-making in the EU [Commission
108 Regulation [EC] Nr. 1881/2006] and United States and China, respectively). However, this
109 mycotoxin is already present in milk and is not produced during cheese storage. Maximum
110 OTA levels are regulated in the EU (Commission Regulation [EC] Nr. 1881/2006) for some
111 foods but not cheese. However, due to its toxicity, OTA was classified by the IARC (1993),
112 as class 2B, possibly carcinogenic to humans. On the basis of the lowest observed adverse

113 effect level (LOAEL) of 8 µg/kg body weight per day for early markers of renal toxicity in
114 pigs (the most sensitive animal species), and applying an uncertainty factor of 450 for the
115 uncertainties in the extrapolation of experimental data derived from animals to humans as
116 well as for intra-species variability, EFSA (2006) established a Tolerable Weekly Intake
117 (TWI) of 120 ng/kg b.w. The majority of *P. verrucosum* isolates are OTA producers and some
118 isolates also produce CIT (Frisvad and Samson, 2004). *Penicillium citrinum* and *P.*
119 *verrucosum* are the only known *Penicillium* species to produce this mycotoxin in cheese
120 (Hymery et al. 2014). CIT, like OTA, is nephrotoxic, but in group 3 as it is not classifiable as
121 to its carcinogenicity towards humans (IARC, 1987). Citrinin is nephrotoxic and a no-
122 observed-adverse-effect level (NOAEL) of 20 µg/kg body weight per day was identified from
123 a 90-day study in rats. Due to the limitations and uncertainties in the database, the derivation
124 of a health-based guidance value was not considered appropriate but a level of no concern for
125 nephrotoxicity of 0.2 µg/kg b.w. per day was determined. Based on the available data a
126 concern for genotoxicity and carcinogenicity could not be excluded at the level of no concern
127 for nephrotoxicity, (EFSA, 2012). Based on toxicity to animals, other *Penicillium* mycotoxins
128 such as penitrem A, a powerful neurotoxin produced by *Penicillium crustosum*, could also be
129 tested for migration in cheese.

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

137

138 2. Materials and Methods

139

140 2.1. *Fungal isolates*

141 All *Penicillia* (belonging to *P. crustosum*, *P. nordicum* and *P. verrucosum*) were isolated
142 from cheese or dairy related environments, with the exception of *P. verrucosum* UBOCC-A-
143 109221, and belong to either the Université de Bretagne Occidentale Culture Collection
144 (UBOCC, Plouzané, France) or were part of the LUBEM laboratory culture collection (Table
145 1). The origin of *P. verrucosum* UBOCC-A-117113 was unknown. These isolates were
146 selected as they belong to the main known *Penicillium* species that can contaminate semi-hard
147 cheeses. All species were previously identified in the laboratory according to partial β -tubulin
148 gene sequencing as described by Ropars et al. (2012). Stock cultures were maintained at -
149 80°C.

150

151 2.2. *Media*

152 For conidial production, Potato Dextrose Agar (PDA, BD Difco, France) was used and plates
153 were incubated at 25°C for 7d after inoculation. For mycotoxin determination, Yeast Extract
154 Sucrose (YES) agar medium (Frisvad and Filtenborg, 1983) buffered at pH 4.5 with
155 phosphate-citrate buffer (Chang et al., 1998) was used as described by Gillot et al. (2017), as
156 this medium is characterized by a high C/N ratio favoring mycotoxin production (Visagie et
157 al., 2014).

158

159 2.3. *Cheese*

160 The French Comté cheese used throughout this study was produced by the Fromagerie Vagne
161 (Poligny, France). All samples were taken from the same circular cheese wheel (batch number

162 FR39.434.002CE). Comté is a semi-hard cheese, ripened for 6 months, with a water activity
163 of 0.95.

164

165 2.4. *Conidia production*

166 Fungal stock cultures (1 ml) were thawed at ambient temperature for about 1h. Plates were
167 then inoculated with 0.1 ml of the stock culture (ca. 1×10^6 conidia.mL⁻¹) and incubated at
168 25°C for 7d. Fresh conidia were harvested using a sterile loop after flooding the surface of the
169 plates with 2 ml of distilled water containing 0.01% Tween 80 (Sigma Aldrich, Saint Quentin
170 Fallavier, France). After counting conidia using a hemocytometer, conidial suspensions were
171 standardized to 1×10^6 conidia.mL⁻¹. Suspensions were used directly after preparation for the
172 different experiments.

173

174 2.5. *Isolate screening for mycotoxin production in YES medium*

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

179 In parallel, fungal growth was also monitored and the corresponding dry weights were
180 correlated to the obtained mycotoxin levels determined by LC-MS/Q-TOF in YES media. For
181 dry weight determinations, 1 μ L of each spore suspension (10^6 conidia.mL⁻¹) were inoculated
182 in the center of YES plates containing sterile cellophane disks (Carrefour, France). Plates
183 were incubated for 7 days at 25°C. After incubation, cellophane disks containing fungal
184 mycelium were carefully removed from the plates and placed into glass Petri dishes. Fungal
185 mycelia were removed from the cellophane disks by adding sterile distilled water. Dry

186 weights were then determined after drying mycelia at 70°C for 48h. Each culture was
187 performed in triplicate.

188

189 2.6. *Isolate screening for mycotoxin production in Comté cheese*

190 Cheese rind was discarded aseptically. Cheese was then cut into 4x4x0.2 cm³ pieces and
191 placed in Petri dishes. Then, 30 µl of a 10⁶ conidia.mL⁻¹ suspension were spread over the
192 surface with a sterile bent glass rod. Petri dishes were placed on a pedestal in hermetically
193 closed plastic containers. To control cheese water activity, a 20% glycerol (w/w) aqueous
194 solution was poured into the bottom of containers. Triplicates were incubated for 10d at 20°C
195 and each cheese piece was stored at -20°C prior to mycotoxin analysis.

196

197 2.7. *Experimental conditions for mycotoxin migration in cheese*

198 The procedure was identical to §2.6 except that cheeses were cut into 4x4x3 cm³ cubes before
199 inoculation (or not for control cheeses) with 30µl of 10⁶ conidia.mL⁻¹. Spore suspensions were
200 evenly spread on each cheese surface before being placed in sterile glass jars. Jars were then
201 placed in larger hermetically closed containers with controlled water activity using the same
202 solution as in §2.6. Triplicates were incubated for 42d at 8°C and 28d at 20°C.

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

210

211 2.8. *Non-selective extraction of fungal metabolites from YES medium*

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

219

220 2.9. *Non-selective extraction of mycotoxins from cheese samples*

221 Cheese samples were aseptically cut into small pieces using a scalpel, placed in 15 mL tubes,
222 then 6.25 mL of diluted ACN (84%) supplemented with 2.5% acetic acid (v/v) per g of cheese
223 were added. Samples were vortexed for 2 min, homogenized with an Ultraturrax T25 digital
224 (IKA, Heidelberg, Germany) for 1 min, then 10 mL hexane were added. Samples were placed
225 on a RotoFlex Plus tube rotator (Sigma, France) for 30 min at room temperature, sonicated for
226 15 min and centrifuged at 4000 g for 10 min at 4°C. After discarding the hexane layer, 5-10
227 mL of the ACN phase were collected and stored at -20°C until QTOF LC-MS analysis.
228 Before analysis, 1 mL of the solution was filtered through a 0.45 µm PTFE membrane syringe
229 4 mm filter (Phenomenex, Torrance, USA) into an amber vial.

230

231 2.10. *Fungal metabolite detection and quantification by QTOF LC-MS*

232 Extracted fungal extrolites, including mycotoxins, were detected and quantified using an
233 Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (QTOF) LC-MS system (Agilent

234 Technologies, Santa Clara, CA, USA). The HPLC system included a Binary pump 1260 and
235 degasser, well plate autosampler set to 10°C and a thermostated column compartment.

236 Mycotoxin standards used for quantification were: citrinin (CIT), ochratoxin A (OTA),
237 penitrem A (PEN A) and roquefortine C (ROQ C). Standards were obtained from Sigma-
238 Aldrich (St Louis, MO, USA) except for PEN A from Cfm Oskar Tropitzsch (Marktredwitz,
239 Germany). All mycotoxin stock solutions were prepared in dimethyl sulfoxide (DMSO) at 1
240 mg.mL⁻¹ and stored at -20°C in amber vials. Calibration curves prepared in ACN ranged from
241 10 to 5000 ng.mL⁻¹. All compound characteristics for mycotoxin identification and
242 quantification are provided in Table 2.

243 For screening isolates for mycotoxin production (YES medium or cheese samples), 2 µL
244 filtered samples were injected and separated using a ZORBAX Extend-C18 column (2.1 x 50
245 mm and 1.8 µm, 600 bar). The column was maintained at 35°C and the flow rate was set to
246 0.3 mL.min⁻¹ using as mobile phase: solvent A (milli-Q water + 0.1% LC-MS formic acid v/v
247 (Carlo Erba Reagents, France) + 0.1% ammonium formate v/v (Thermo Fisher Scientific,
248 Waltham, MA, USA)) and solvent B (100% LC-MS grade ACN). Solvent B was maintained
249 at 10% for the first 3 minutes, followed by a gradient of 10-100% of B for 42 min. Finally,
250 solvent B was maintained at 100% for 5-min post-time. Analytes were ionized in both ESI+
251 and ESI- modes (ESI: Electrospray Ionization). Mass spectrometer conditions were: capillary
252 voltage, 4.0 kV; source temperature, 325°C; nebulizer pressure, 50 psig; drying gas, 12 L/min
253 and ion range, 100 to 1000 m/z. Mycotoxin detection and identification was performed using
254 retention time values ± 1 min and the corresponding quantifier or qualifier ions for each
255 mycotoxin standard listed in Table 2. Mycotoxin concentrations were calculated from the
256 equation $y = mx+b$, as determined by weighted ($1/x^2$) linear regression of the matrix-matched
257 calibration data. For YES samples, these values were also correlated to the calculated fungal
258 biomass (determined dry weight values) and specific mycotoxin production was expressed as

259 ng per g dry weight. In contrast to YES medium, mycotoxin production was expressed as ng
260 per g cheese for Comté cheese.

261 For mycotoxin migration experiments in Comté cheese, some changes were made to optimize
262 the quantification of the two target mycotoxins CIT and OTA as follows: 2 μ L filtered
263 samples were injected and separated in a Poroshell 120 EC-C18 column (2.1 x 50 mm and 2.7
264 μ m, 600 bar) maintained at 35°C. The flow rate was set to 0.3 mL.min⁻¹ using as mobile
265 phase: solvent A (milli-Q water + 0.1% LC-MS formic acid v/v + 0.1M ammonium formate
266 (Carlo Erba Reagents, France)) and solvent B (100% LC-MS grade CAN + 0.1% LC-MS
267 formic acid v/v (Carlo Erba Reagents, France)). Solvent B was maintained at 10% for the first
268 4 minutes, followed by a gradient of 10-100% of B for 16 min. Finally, solvent B was
269 maintained at 100% 2 min followed by 5 min post-time. Target analytes were ionized in ESI+
270 mode and identical mass spectrometer conditions were used as described above. Mycotoxin
271 detection and identification was performed as above and specific mycotoxin production was
272 expressed as ng per g cheese.

273 Method performance and validation parameters including linearity (R^2), detection limit (DL),
274 quantification limit (QL), recovery values and matrix effects were also assessed for target
275 metabolites of interest according to the ICH guidelines (ICH Harmonized Tripartite
276 Guideline, 2005). The calibration method used for reliable metabolite quantifications was
277 prepared as previously described (Fontaine et al., 2015). Briefly, blank matrix (Comté cheese)
278 aliquots were treated using the sample preparation procedure described above to obtain blank
279 extracts. Each blank extract was then spiked with an adequate volume of the stock solutions
280 of the two target mycotoxins, CIT and OTA, to reach concentrations ranging from 5 to 5000
281 ng.mL⁻¹. Calibration curves were obtained by plotting the peak area against mycotoxin
282 concentrations using a $1/x^2$ weighted linear regression model. According to ICH guidelines
283 (ICH Harmonized Tripartite Guideline, 2005), DL and QL of each metabolite was obtained

284 by multiplying the standard deviation of y-intercepts of regression lines divided by the slope,
285 by 3.3 and 10, respectively (Table 2). Recovery percentage was evaluated in triplicate using
286 cheese matrices spiked with a mix of CIT and OTA at 250 and 2500 ng.g⁻¹ (recovery >85%
287 for both concentrations). Finally, all calibration curve calculations were carried out using the
288 Agilent MassHunter Workstation Software (Agilent Technologies, Santa Clara, CA, USA).

289

290 **3. Results**

291

292 *3.1. Screening of Penicillium isolates for mycotoxin production*

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

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

309 levels of OTA and CIT in Comté cheese (Figure 1b). Therefore, this isolate was selected to
310 determine the mycotoxin migration in a cheese substrate.

311

312 3.2. Migration of mycotoxins in Comté cheese

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

318 At 8°C, production of CIT and OTA started after 14 and 28 days incubation, respectively
319 (Figure 2a). At 14 days, CIT was mainly detected in 2mm depth samples with and without
320 fungal mycelium although highest concentrations were detected when the mold was present.
321 Low CIT concentrations (about 125 ng/g) were also detected from 2-4mm in depth. At 21
322 days, significant amounts of CIT, about 1500 ng/g and 1000 ng/g, were detected in the 0-2
323 mm and 2-4 mm slices, respectively, as well as very low levels between 4-6mm (<75 ng/g).
324 At 28 days, CIT was detected at concentrations ranging from 1100 to 500 ng/g up to 6mm
325 depth, as well as at about 50 ng/g up to 8mm depth, but the concentrations at the cheese
326 surface decreased, thus suggesting that the production of CIT at the surface of the cheese had
327 stopped and consequently that CIT had migrated into the depth of the cheese. At 28 and 35
328 days, OTA was produced at concentrations reaching about 250 ng/g and 850 ng/g,
329 respectively, and detected up to 6mm in depth by 35 days. At both 35 and 42 days, OTA
330 production was higher than CIT and by 42 days, OTA concentrations reached maximum
331 levels with up to about 3500 ng/g cheese. At this time, OTA was detected at a depth greater
332 than 10mm. Mycotoxin production was then correlated to changes in fungal growth after
333 incubation at 8°C (Figure 3a). Up to 14 days, loose white mycelium was observed on the

334 surface of cheeses incubated at 8°C (Figure 3a). By 21 days, a blue color due to the
335 production of conidia appeared. Then, from 28 to 42 days, blue mold completely covered the
336 cheese surface which corresponded to the same time period when OTA was produced.

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

347 Fungal growth at 20°C was characterized by white mycelium largely covering the cheese
348 surface after 7 days incubation at 20°C (Figure 3b). Blue mold aspect due to conidia
349 production was observed from day 14 only at 20°C versus day 21 for 8°C (Figure 3a). A
350 dense blue mold layer was then visible at 21d and 28d incubation at 20°C. Highest OTA and
351 CIT quantities were produced when blue mold was visible on the cheese surface.

352

353 **4. Discussion**

354

355 Mycotoxin production profiles were first determined by QTOF LC-MC for 7 isolates
356 belonging to 3 known mycotoxin producing *Penicillium* species - *P. nordicum*, *P. verrucosum*
357 and *P. crustosum* - that can contaminate dairy foods using two different substrates, YES agar
358 and Comté cheese. Our results showed that, in some cases, different mycotoxins were

359 produced depending on the growth substrate used to cultivate the fungal isolates. This
360 observation has already been described for the same 3 *Penicillium* species using 3 substrates
361 by Kokkonen et al. (2005). ROQ C and PEN A were synthesized on both substrates by all 3
362 *P. crustosum* isolates. These species have already been described to produce these compounds
363 in different substrates including foods (Kokkonen et al., 2005; Leggierei et al., 2017; Olsen et
364 al., 2017; Prencipe et al., 2018). ROQ C and PEN A were produced by *P. verrucosum*
365 UBOCC-A-117113 on YES agar but not in cheese. It was reported that this species was able
366 to produce ROQC, (Kozlovsky and Reshetilova, 1984), but this result was later suggested to
367 be incorrect by Frisvad et al (2004). *Penicillium verrucosum* var. *cyclopium* RV 67718
368 isolated from ground cassava collected in Burundi, was cultivated under laboratory conditions
369 to produce the mycotoxin roquefortine C (Musuka et al., 2004). But the production of PEN A
370 by *P. verrucosum* was never reported before. *P. verrucosum* UBOCC-A-117113 and
371 UBOCC-A-111269, identified by the β -tubulin gene, were closely related and clearly
372 identified as *P. verrucosum*. In our opinion, this result was not due to a misidentification, but
373 it should be confirmed by other research groups.

374 OTA and CIT synthesis were highly variable according to substrate and fungal isolate. *P.*
375 *nordicum* synthesized OTA in cheese but not in YES medium. This mycotoxin has already
376 been shown to be synthesized at variable concentrations by this species on YES, cheese and
377 bread analogues (Kokkonen et al., 2005) and also according to NaCl content of the substrate
378 (Schmidt-Heydt et al., 2012). The amount of CIT produced by *P. nordicum* in YES medium
379 was below the DL. Finally, 2 *P. verrucosum* isolates were the strongest OTA producers in
380 cheese and 1 isolate also simultaneously produced CIT on this substrate while extracts from
381 YES medium exclusively contained only 1 mycotoxin at a time. The OTA+CIT producing *P.*
382 *verrucosum* isolate was therefore further studied in our model cheese system to evaluate the
383 potential migration of these compounds into the cheese. Noteworthy, our results highlighted

384 the importance to correctly choose a substrate to determine mycotoxin production profiles of
385 a given strain. We recommend testing more than one substrate to determine whether a fungal
386 strain is able to produce one or multiple mycotoxins is therefore highly recommended.

387 *P. verrucosum* grows from 0 to 31°C, with an optimum at 20°C (Pitt and Hocking,
388 2009). Accordingly, it was observed in our study that the development of this species on
389 Comté was faster at 20°C than at 8°C. The increase of temperature from 15 to 20°C with
390 respect to the same incubation time resulted in an increase in OTA biosynthesis by a factor of
391 10 (Battilani et al., 2010). In contrast to that study, the concentration of OTA produced in
392 Comté at 8 and 20°C were not significantly different. The ability to biosynthesize OTA and
393 CIT at 10°C was not detected on solid cereal substrates (Wawrzyniak and Waśkiewicz, 2014),
394 whereas production of OTA and CIT by *P. verrucosum* was reported on YES medium at 6°C
395 (Takahashi and Yazaki, 2007). *P. verrucosum* grew and produced CIT at 20°C and 25°C, but
396 not at 5°C, 30°C, or 35°C on MEA, CYA, and YES agar (Heperkan et al., 2009). CIT
397 biosynthesis in *P. verrucosum* was shown to be highly regulated by environmental factors like
398 temperature, water activity or pH (Touhami et al. 2018). A higher level of CIT *versus* OTA
399 was produced by *P. verrucosum* at 6°C on YES medium (Takahashi and Yazaki, 2007).
400 Recent publications suggested that the concentration of CIT may be high in cereals and may
401 even exceed that of OTA (Vrabcheva et al., 2000; Meister, 2004; Zaied et al., 2012;
402 Kononkenko and Burkin, 2013, Wawrzyniak and Waśkiewicz, 2014). On Comté cheese,
403 maximum amounts of OTA and CIT at 8°C were of the same order of magnitude although
404 produced at different times, whereas the amounts of CIT were about 10 fold that of OTA at
405 20°C. The latter observation strengthened this assumption.

406 In the present study, the maximum OTA concentration found in Comté cheese was
407 about 3 mg/kg. This amount may depend on the isolate, but also on the substrate. For example
408 OTA has been found in spoiled cheeses, at up to 7 mg/kg (Jarvis, 1983), in moldy

409 cheesecakes, at up to 1 mg/kg (Piskorska-Pliszczynska and Borkowska-Opacka, 1984) and in
410 traditional raw cow milk hand-made semi-hard cheeses at levels ranging from 18-146 µg/kg
411 in the cheese interior and 1-262 µg/kg in the rind (Pattono et al., 2013). Up to 600 mg/kg of
412 CIT was also produced on fresh goat cheese by *P. citrinum* after 10 days incubation at 20°C
413 (Bailly et al., 2002). In the present study, 2.5 mg/kg and 45 mg/kg of CIT were detected in
414 Comté at 8 and 20°C, respectively, although in this cheese, CIT production was not due to *P.*
415 *citrinum* but *P. verrucosum*.

416 *P. nordicum* is adapted to NaCl-rich environments like hypersaline waters (Butinar et
417 al., 2011) or NaCl-rich food products like cheeses or dry-cured meats (Larsen et al., 2001).
418 Important habitats of *P. verrucosum* are cereals and cereal products; however, this species can
419 also occur on salt-rich foods, like dry-cured meats and cheeses, and is obviously be
420 responsible for the co-occurrence of OTA and CIT in these types of products (Markov et al.,
421 2013). As expected, the *P. nordicum* isolate tested in our study developed well and produced
422 OTA in Comté, but did not produce any mycotoxins in YES medium. It was reported that
423 strains of *P. verrucosum* exclusively produced CIT on YES, whereas both OTA and CIT were
424 detected in considerable amounts on a bread analogue (Kokkonen et al., 2005). Similarly, the
425 *P. verrucosum* isolate selected in our study for its ability to produce OTA and CIT on Comté
426 cheese, only produced CIT on YES medium.

427 In *P. verrucosum*, CIT and OTA biosynthesis are mutually regulated (Schmidt-Heidt
428 et al., 2011). Both mycotoxins are polyketides with a highly similar dihydrocoumarin moiety,
429 but in the case of OTA, this moiety contains a chlorine atom and is coupled to the amino acid
430 phenylalanine. High amounts of one of these secondary metabolites are produced at the
431 expense of the other and this species can likely adapt its biosynthesis depending on the
432 encountered environmental conditions in a given habitat. NaCl concentration plays an
433 important role in OTA biosynthesis which can certainly be related to the chlorine atom in its

434 chemical structure. It was suggested that OTA biosynthesis helps ensure chloride homeostasis
435 in the cell in adaptation to a NaCl-rich environment (Schmidt-Heidt et al., 2012). Shift from
436 CIT towards OTA biosynthesis was also reported at increasing NaCl concentrations
437 (Schmidt-Heidt et al., 2012; Giesen et al, 2017). High NaCl concentrations could cause
438 increased intracellular osmotic stress due to intensive mitochondrial respiration (Petrovic,
439 2006), therefore the antioxidant properties of CIT may also impact adaptation to such an
440 environment (Giesen et al, 2018). At 8°C, the shift from CIT towards OTA could not be
441 explained by an increasing NaCl concentration, due for example to drying of the surface of
442 the cheese, because water activity in the incubation environment of Comté cheeses was
443 controlled. A change in the pH in Comté cheese, due for example to lipid or protein
444 degradation by the fungus, could explain the shift from CIT to OTA at 8°C. Also, protein
445 degradation could provide free amino acids, including phenylalanine, in the environment
446 required for OTA biosynthesis. The absence of this shift at 20°C on Comté cheese could be
447 explained by faster degradation or modification of environmental factors at the surface of
448 Comté, especially as fungal development was very intense on the cheese surface under these
449 conditions.

450 No study reported that CIT could be an OTA precursor. It rather appears that these two
451 mycotoxins are mutually regulated. Therefore, the decrease in CIT observed after 14 days
452 incubation at 8°C was not due to a transformation into OTA. The decrease in CIT could rather
453 be explained, at least partially, by the diffusion of this mycotoxin into the cheese. In fact, by
454 considering the first 4mm at 21 days incubation, the amount of CIT was about 5000 ng (the
455 weight of each 2mm slice was about 2g), as compared to 800 ng at 35 days incubation. By
456 comparison, CIT produced by *P. citrinum* on black olives was maximum at 40 days
457 incubation at 20, 25, and 30°C than decreased until 60 days (Heperkan et al. 2012). In another
458 study on cheese, it was suggested that the disappearance of this toxin was due to a chemical

459 reaction with cheese components and not to any microbial action (Bailly et al. 2002). Since
460 cheeses are high in casein, it is possible that reactive groups in the protein react with the
461 mycotoxin, causing its disappearance. Previous data have also demonstrated that CIT may be
462 inactivated by cysteine and can be bound to albumin and plasma proteins (Scott, 1977).

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

471

472 **5. Conclusions**

473

474 In the farm to fork approach, the consumer is an important element of the chain. Regulations
475 can be enforced at the different steps from production to retail, but recommendations can only
476 be suggested to the consumer. With respect to moldy food products, these recommendations
477 are scarce, not easily available and not supported by many scientific experiments. In order to
478 ensure food safety, it should be recommended to discard sell by date products stored in the
479 fridge because mold development can hide a contamination by pathogenic bacteria. Therefore,
480 this study focused on Comté cheese that has not been reported to be contaminated by such
481 bacteria during storage. It should be underlined that the conclusions of the present study
482 should not be extrapolated to other cheeses, especially soft ones. The approach described in
483 the present study was also based on insufficient knowledge of the consumer on mold

484 contamination, i.e., the consumer cannot distinguish a harmless fungi (e.g., Mucoromycota)
485 from potentially toxigenic species. It should also be bared in mind that many people are
486 unaware of mycotoxins, and that mycotoxins can migrate in food products.

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

496 Considering mycotoxins as chemical hazards is consistent with the objective of setting
497 international regulatory standards based on toxicological studies (Dantigny and Bensoussan,
498 2013). However, this approach was not suitable to improve food safety at the consumer level
499 simply because mycotoxins cannot be quantified by the consumer. Simple recommendations
500 should be based on the visual aspect of cheese regardless of storage time because many
501 consumers do not know how long food products are stored. Due to slower growth and lower
502 mycotoxin productions at 8°C than at 20°C, it can also be recommended to the consumer to
503 store Comté cheese at refrigerated temperatures.

504

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506

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725

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

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

727

728

729 Table 2. Target mycotoxin quantification parameters used for QTOF LC-MS analyses. RT:
 730 retention time (*RT values obtained for isolate screening), MW: molecular weight, ESI:
 731 electrospray ionization, DL: detection limit, QL: quantification limit.
 732

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 ₅ O ₂	15.1*	389.5	390.1	na	+	136.8	414.5	0.98

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737 Figure captions

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739 Figure 1: Specific mycotoxin production by seven *Penicillium* species isolates after 7d growth at 25°C on YES
740 medium (A) or 10d growth at 20°C on 6 month ripened Comté cheese (B). Quantified mycotoxins: CIT (■),
741 OTA (■), PEN A (□) and ROQ C (□).

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744 Figure 2 : CIT (■) and OTA (■) quantification in Comté cheese, inoculated with *P. verrucosum* UBOCC-A-
745 111269, after 42 and 28 days incubation at 8°C (A) and 20°C (B), respectively. d: day; D0: 0-2mm depth with
746 mould, D1: 0-2mm depth without mould, D2: 2-4mm depth, D3: 4-6mm depth, D4: 6-8mm depth, D5: 8-10mm
747 depth, D6: 10-12mm depth, D7: 12-14mm depth, D8: 14-16mm depth.

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749 Figure 3: Weekly monitoring of fungal growth on semi-hard Comté cheese during incubation at 8°C (A) and
750 20°C (B)

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Figure captions

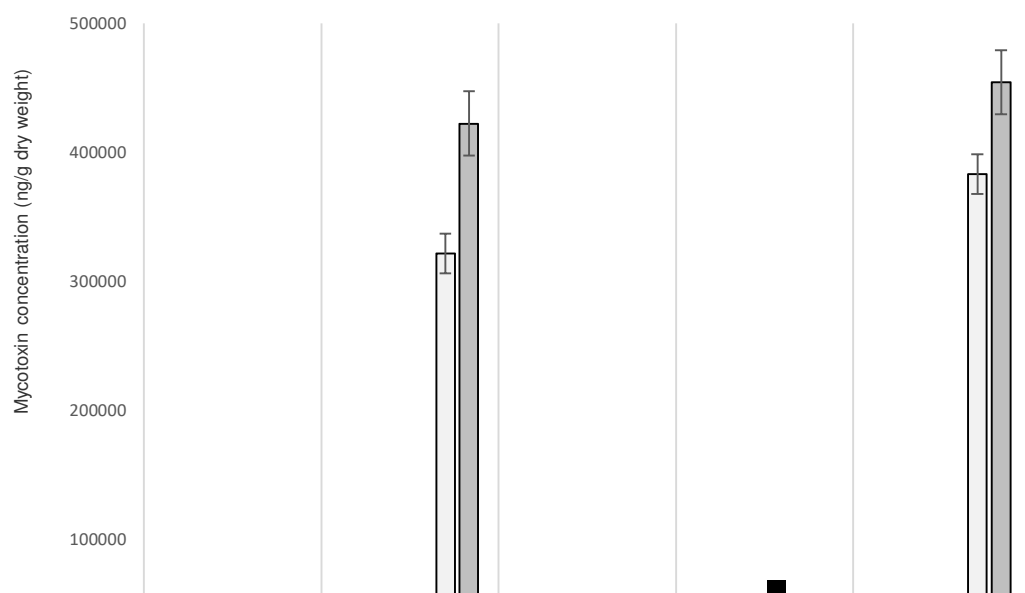
Figure 1: Specific mycotoxin production by seven *Penicillium* species isolates after 7d growth at 25°C on YES medium (A) or 10d growth at 20°C on 6 month ripened Comté cheese (B). Quantified mycotoxins: CIT (■), OTA (■), PEN A (□) and ROQ C (□).

Figure 2: CIT (■) and OTA (■) 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: 0-2mm depth with mould, D1: 0-2mm depth without mould, D2: 2-4mm depth, D3: 4-6mm depth, D4: 6-8mm depth, D5: 8-10mm depth, D6: 10-12mm depth, D7: 12-14mm depth, D8: 14-16mm depth.

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

Figure 1.

A



B

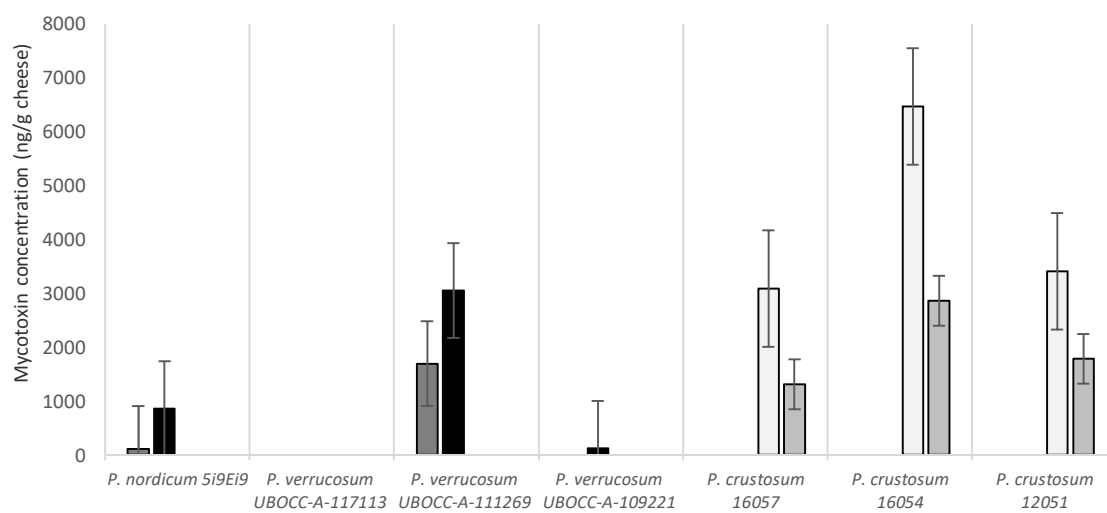
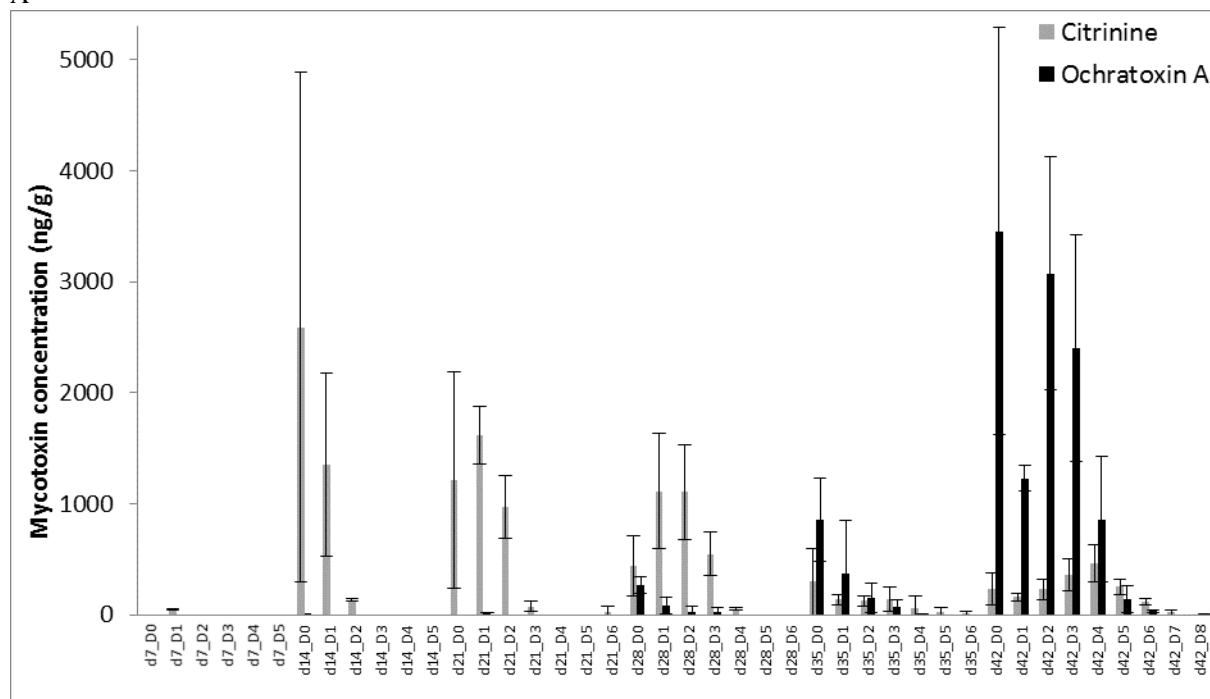


Figure 2.

A



B

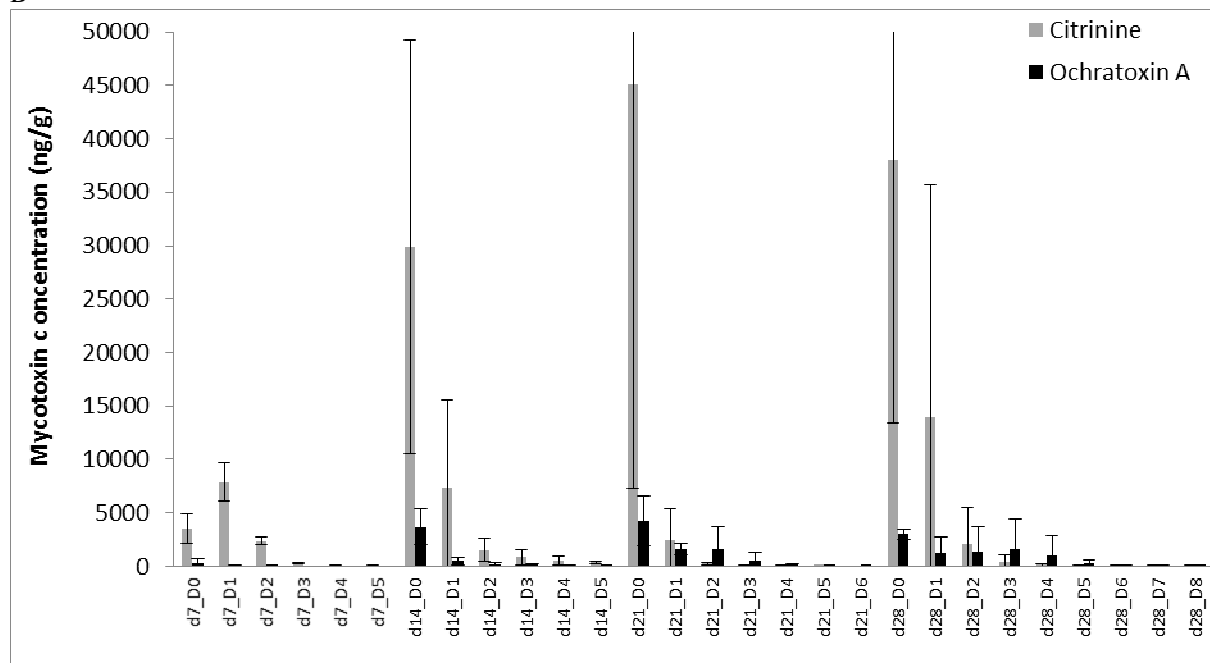
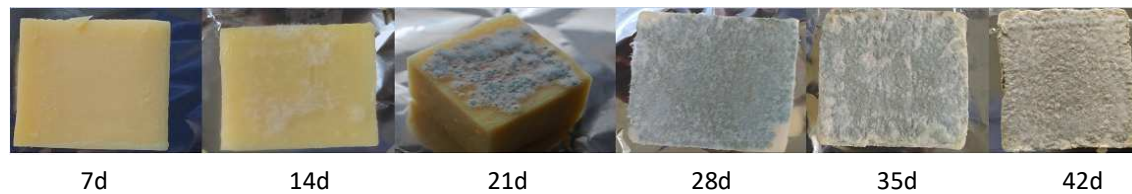


Figure 3.

A



B

