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1 **Impact of intraspecific variability and physiological state on *Penicillium commune***
2 **inactivation by 70% ethanol.**

3

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16 **Keywords: mold, conidia, fungicide, disinfectant, predictive mycology**

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Abstract

The aim of this study was to assess the impact of the physiological state and intraspecific variability on the efficacy of 70% ethanol to inactivate conidia of *Penicillium commune*, used as a representative species of dairy product contaminants. Four physiological states were obtained by modifying the water activity during the production of conidia (0.995 and 0.950) and the harvesting conditions (hydrated and non-hydrated). These conditions were applied to four different *P. commune* strains isolated from contaminated dairy products. Five minutes exposure to 70% ethanol at ambient temperature allowed total inactivation of conidia ($> 4 \log_{10}$) regardless of the physiological state or the strain. For 1 min exposure, regardless of the strains, only dry-harvested conidia produced at a_w 0.950 exhibited survivors. Survival after 2 min exposure was observed for this physiological state for *P. commune* UBOCC-A-116003 only. For this strain, the impact of the physiological state was greater than $1.54 \log_{10}$ between dry-harvested conidia produced at a_w 0.950 that exhibited survivors after 1 min treatment and the 3 other kinds of conidia that were all inactivated. For 1 min exposure, by comparing the more resistant strain to the three other strains, the impact of the intraspecific variability was $2.35 \log_{10}$. These results demonstrated that the physiological state of the conidia, the representativeness of the tested species and strains should be taken into account to assess the efficacy of disinfectants in dairies.

41

42 **1. Introduction**

43 Fungi are a major source of contamination in agri-food industrial environments due to their
44 ability to grow on a large number of food matrices such as dairy products, bakery products,
45 cereals, vegetables, meats, processed products, high-sugar products and oilseed (Dagnas and
46 Membre, 2013; Filtenborg et al., 1996). Development of spoilage molds on these products
47 impacts their organoleptic properties, and causes food waste and economic losses. Although it
48 is difficult to get the exact figures, it is estimated that 5 to 10% of global food losses and
49 waste are attributed to spoilage fungi (Pitt and Hocking, 2009). Among those, molds are able
50 to easily disseminate in processing plant through the production of conidia by asexual
51 reproduction (Salustiano et al., 2003). Airborne conidia are produced in large quantities,
52 facilitating mold dissemination in the processing plant and on equipment, thus leading to food
53 contamination (Heldman, 1974).

54 To prevent the risk of contamination, manufacturers use a wide variety of chemical surface
55 disinfectants. Liquid disinfectants that contain ethanol as the fungicide molecule are sprayed
56 on machine surfaces that can be in contact with food products. In Europe, the fungicidal effect
57 of disinfectants is defined by the European standard EN 13697 (2001). While this standard is
58 widely used, three major limits can be highlighted.

59 Firstly, *Aspergillus brasiliensis* (ATCC 16404) and *Candida albicans* (ATCC 10231) are
60 the reference species for testing disinfectants for their fungicidal activity. However,
61 depending on the considered type of food production, these species may be not representative
62 of the encountered contaminants. For example, species from the *Penicillium* genus would be
63 more representative of molds responsible for cheese spoilage (Ledenbach and Marshall,
64 2009). Among them, *Penicillium commune* is often isolated from spoiled dairy products
65 (Filtenborg et al., 1996; Garnier et al., 2017; Hocking and Faedo, 1992). The choice of the
66 fungal spoiler representative of contamination is important because the efficacy of a

67 disinfectant depends greatly on the considered species. For example 0.15% peracetic acid
68 decreased the population of *A. brasiliensis*, *C. albicans* and *P. commune* (CCT 7683) by 0.67,
69 1.3 and 2.3 log₁₀, respectively (Bernardi et al., 2018). These authors showed that the
70 difference between the species was even more pronounced at 1.5% peracetic acid as these
71 species exhibited decrease of 3 log for *A. brasiliensis* and more than 5 log for *C. albicans* and
72 *P. commune*.

73 Secondly, the use of only one strain of the selected species can raise questions concerning
74 its representativeness of the species behavior towards the studied factor (*i.e.* biocide resistance
75 or sensitivity). Several studies have shown intraspecific differences on inactivation for a given
76 biocide (Bundgaard-Nielsen and Nielsen, 1996; Korukluoglu et al., 2006; Salo and Wirtanen,
77 2005). For example, the application of 1.5% benzalkonium chloride for 10 min at 20 °C
78 inactivated 1.2 and 4 log₁₀ *Aspergillus versicolor* conidia depending on the 2 studied strains
79 (Bundgaard-Nielsen and Nielsen, 1996).

80 Finally, in these standards, conidia are hydrated to obtain conidial suspension. However,
81 this condition may not represent the physiological state of airborne conidia present in an
82 industrial environment. Moreover, it is unlikely that conidia are produced under optimal
83 conditions in dairies. It is suggested that molds may have rather encountered various abiotic
84 stresses before and during the production of conidia. It has been shown previously that
85 inactivation of conidia depended greatly on the strain, the physiological state, and the medium
86 used (Dantigny, 2016). Water activity (a_w) during the production of conidia is an essential
87 factor that impacts the physiological state of conidia. It was shown to play an important role
88 in many biological responses in fungi such as germination, mycotoxin production, and
89 resistance to heat and chemical stresses (Dantigny, 2016; Dantigny and Nanguy, 2009). Dao
90 and Dantigny (2009) showed that a reduced a_w during conidial production was associated with

91 a greater resistance of *Penicillium chrysogenum*, *Penicillium digitatum* and *Penicillium*
92 *italicum* conidia to ethanol vapors (0.67 kPa).

93 In this context, this study aimed at assessing the impact of the intraspecific variability and
94 the physiological state on the efficacy of 70% ethanol solution to inactivate four *Penicillium*
95 *commune* strains. For each tested strain, 4 types of conidia, corresponding each to a different
96 physiological state, were obtained by modifying the water activity during conidial production
97 (0.950 a_w and 0.995 a_w) and harvesting conditions (hydrated and non-hydrated).

98

99 **2. Materials and methods**

100 *2.1. Molds*

101 The four studied *P. commune* strains were provided by the University de Bretagne
102 Occidentale Culture Collection (UBOCC). They were all isolated from contaminated cheeses,
103 their source is listed in Table 1. The molds were maintained on Potato Dextrose Agar (PDA)
104 medium at 4 °C.

105

106 *2.2. Conidial production*

107 Conidia were produced from mycelium grown on PDA at 2 a_w levels (0.995 and 0.950) for
108 7 days at 25 °C. a_w was adjusted by substituting a part of the water with an equal weight of
109 glycerol. The relative amount of glycerol was 20.6% (w/w) to obtain PDA media at a_w 0.950.
110 The a_w value of the culture media was controlled after autoclaving and solidification at 20 °C
111 using a Tunable Diode Laser aw-meter (TDL Aqualab, Decagon Devices, Pullman, WA,
112 USA) with an accuracy of 0.005 a_w unit.

113

114 *2.3. Conidia harvesting protocols*

115 Dry-harvested conidia were obtained mechanically without any contact with water
116 according to Dao and Dantigny (2009). Ten sterile glass beads (diameter 3 mm) were
117 deposited on the agar surface and the Petri dish was shaken gently to detach the conidia.
118 Then, the plate was turned upside-down and the conidia were harvested on the lid by gently
119 tapping 100 times on the bottom of the Petri dish.

120 Hydrated conidia were obtained by flooding the lid with 5 ml of sterile saline solution
121 (NaCl, 9 g/l of water) containing Tween 80 (0.015% v/v). For this condition, conidia were
122 hydrated for 30 minutes before applying the disinfectant testing procedure.

123

124 2.4. *Disinfectant testing procedures*

125 Seventy percent ethanol solution was prepared from anhydrous ethanol diluted in distilled
126 water containing Tween 80 (0.015%, v/v). Treatment of dry harvested conidia began when 10
127 ml of a 70% ethanol solution was applied to the dry-conidia on the lid. Before the first
128 sampling time, the suspension (ethanol containing conidia) was transferred to a Falcon tube
129 and homogenized by vortexing for 10 s. Treatment of hydrated conidia began when 1 ml of
130 conidial suspension was transferred in 9 ml of a 78% ethanol solution. After homogenization
131 by vortex (10 s), the final ethanol concentration of the mixture was 70%. Disinfection tests
132 were performed at ambient temperature, 20 ± 1 °C.

133 After 1, 2 and 5 minutes of exposure, ethanol was neutralized by diluting ten-fold the
134 suspension in a saline solution (NaCl, 9 g/l of water) containing Tween 80 (0.015% v/v). The
135 initial amount of dry-harvested conidia (N_0) was evaluated by counting on a Malassez cell
136 according to Dao et al. (2008). The method was based on the principle that the application of
137 ethanol did not affect the morphology of the conidia during the time of the experiment so it is
138 possible to count under the microscope the initial conidia treated even after the experiment
139 was started.

140

141 2.5. Viability assessment

142 After neutralization, the treated conidia were grown on PDA at 25 °C for 3 days by
143 spreading 100 µl of sample and 4 subsequent decimal dilutions. If no visible mycelium was
144 observed after 3 days, the dishes were incubated for 4 additional days. Only counts (N_t) in the
145 range 10 to 100 colonies per plate were considered. Conidial inactivation was expressed as the
146 logarithmic reduction factor $\log_{10} (N_t/N_0)$.

147

148

149 3. Results

150 \log_{10} inactivation values of conidia of the 4 studied *P. commune* strains exposed to 70%
151 ethanol for 1, 2 and 5 minutes are shown in Table 2. The obtained data showed that after five
152 minutes exposure to 70% ethanol, total inactivation of conidia was achieved, regardless of the
153 considered strain, a_w for conidial production and harvesting conditions. For all strains, after 1
154 minute exposure, only dry-harvested conidia produced at a_w 0.950 exhibited survivors. Under
155 these production and harvesting conditions, *P. commune* UBOCC-A-116003 was the most
156 resistant with an inactivation value of 2.35 \log_{10} . The three other *P. commune* strains had
157 inactivation values in the range 4.62 \log_{10} to 5.40 \log_{10} . In addition, *P. commune* UBOCC-A-
158 116003 was the only one to survive at 2 minutes exposure with an inactivation value of 3.89
159 \log_{10} (Table 2) and even at 3 min (inactivation value of 4.78 \log_{10}) (data not shown).

160 Two different groups can be distinguished according to the inactivation data obtained for 1
161 min treatment. A “weak” physiological state group (*i.e.* hydrated conidia, regardless of the a_w
162 used for conidia production, and non-hydrated conidia produced at a_w 0.990), for which all
163 conidia were inactivated, and a “strong” physiological state group (*i.e.* non-hydrated conidia
164 produced at a_w 0.950) for which some conidia remained viable. All conidia from the “weak”

165 physiological state were inactivated, inactivation was greater than 3.86, 3.85, and 4.02 log₁₀
166 for UBOCC-A-119012, UBOCC-A-112050 and UBOCC-A-112059, respectively. The
167 number of conidia obtained for the conditions of the “weak” physiological state group was
168 lower than that the one produced for the “strong” physiological state with the exception of
169 UBOCC-A-112059. Because more conidia were obtained for the “strong” physiological state,
170 inactivation seemed greater than for the “weak” one. In fact, the effect of the physiological
171 state can be accurately determined for the strain UBOCC-A-116003, as all conidia from the
172 “weak” physiological state group were inactivated. Inactivation was greater than 3.89 log₁₀
173 for this group, whereas the “strong” group exhibited only a 2.35 log₁₀ inactivation value. In
174 this case, the difference between the two groups was greater than 1.54 log₁₀.

175 The impact of intraspecific variability in *P. commune* could be quantified for the “strong”
176 physiological state only. A group of “sensitive” strains, namely UBOCC-A-112050, UBOCC-
177 A-112059 and UBOCC-A-119012 could be distinguished from the “resistant” UBOCC-A-
178 116003 strain. For 1 min treatment, the difference between the inactivation value of the
179 UBOCC-A-116003 and the mean of the 3 “sensitive” strains was 2.61 log₁₀.

180

181 **4. Discussion**

182 In this study, 5 minutes exposure allowed total inactivation (inactivation values greater
183 than 4 log₁₀) of conidia of *P. commune* regardless of the tested strain, and the production or
184 harvesting conditions. Despite little information in the literature on the susceptibility of *P.*
185 *commune* to ethanol, our results are in agreement with those of Bundgaard-Nielsen and
186 Nielsen (1996) who observed the absence of growth of three *P. commune* strains within 14
187 days after an exposition to 70% ethanol solution for 10 minutes.

188 The choice of a species representative of the actual contamination of a given food sector,
189 food product and even plant is an important question. Indeed, according to standards, the

190 fungicidal activity of a disinfectant must be evaluated on the two reference strains belonging
191 each to the *A. brasiliensis* and *C. albicans* species. However, these species might not be the
192 most representative contaminants of a studied food sector. In this context, Bernardi et al.,
193 (2018) showed that these 2 strains behaved differently than 4 actual food contaminant strains
194 (belonging to 4 different species, including *P. commune*) when exposed to 5 disinfectants
195 (benzalkonium chloride, biguanide, peracetic acid, quaternary ammonium and sodium
196 hypochlorite). Moreover, sensitivity variability was observed between the 4 contaminant
197 species (*Cladosporium cladosporioides*, *P. commune*, *Penicillium polonicum* and *Penicillium*
198 *roqueforti*) representative strains. These observations underline the importance of the initial
199 choice of the species to be tested that might allow optimizing disinfectant procedure and
200 potentially reduce their concentrations, the use of water for rinsing and therefore the
201 associated cost, while guarantying safety.

202 Concerning the representativeness of the tested strains, in this study, the time necessary to
203 inactivate 10^4 *P. commune* conidia with 70% ethanol varied according to the considered
204 strain. It was less than 1 min for the UBOCC-A-119012, UBOCC-A-112050 and UBOCC-A-
205 112059 strains, and greater than 2 min (and even 3 min -data not shown-) for the UBOCC-A-
206 116003 strain. The effect of the fungal strain on the susceptibility to a disinfectant was
207 previously reported (Bernardi et al., 2019; Korukluoglu et al., 2006). Korukluoglu et al.
208 (2006) described disinfectant susceptibility of 5 strains of *Aspergillus niger* and *Penicillium*
209 *roqueforti* to 8 disinfectants and different concentrations. Depending on the strain, the time to
210 inactivate 10^6 *A. niger* conidia using isopropyl alcohol ranged from 10 to 25 min, while that
211 time varied between 5 and 16 min to inactivate 10^6 *P. roqueforti* conidia using 0.3% peracetic
212 acid. The observed intraspecific susceptibility was dependent on the disinfectant and the
213 concentration used. Bernardi et al. (2019) also showed evidence of strain-dependent
214 inactivation. When applying 3% peracetic acid for 15 min on 3 *P. roqueforti* strains, the

215 inactivation values were 3.69, 3.70 and 5.66 \log_{10} with 1.97 \log_{10} difference observed
216 between the sensitive strain and the 2 other resistant strains.

217 Concerning the impact of the physiological state on inactivation, our results clearly showed
218 that production of conidia and harvesting conditions have a strong impact on the survival to
219 the disinfectant treatment. In the tested conditions, dry-harvested conidia produced at a_w 0.950
220 survived after 1 minute exposure. These conditions could be considered as more
221 representative of the processing plant than the standard conditions. First, in the dairy industry,
222 fungal contamination of food products is due to airborne conidia that are disseminated in the
223 air (Burgain and Dantigny, 2016; Salustiano et al., 2003). Secondly, it is very unlikely that
224 conidia are produced in dairy plants under optimum a_w conditions. As suggested by previous
225 studies, as compared to a_w 0.995, a reduced a_w induced major changes in the physiological
226 state and in the resistance of conidia to ethanol (Dao and Dantigny, 2009; Nesci et al., 2004).
227 In our study, it was not possible to determine whether a_w during production of conidia was
228 more important than the harvesting conditions on inactivation. In fact, compared to hydrated
229 conidia produced at a_w 0.995, the effect of modifying one condition only, either the harvesting
230 condition or the a_w during conidial production, could not be quantified as these 3 conidia
231 types were inactivated. The impact of the physiological state was also tested on 3 *Penicillium*
232 species exposed to ethanol vapors (Dao and Dantigny, 2009). As compared to dry-harvested
233 conidia that remained unaffected by ethanol treatments, hydrated conidia exhibited
234 inactivation in the range 1 to 3.5 \log_{10} depending on species and treatment conditions. The
235 effect of a_w during production of conidia on inactivation appeared even greater, after 48h
236 treatments with 0.67 kPa ethanol vapors, the difference between conidia produced at 0.99 and
237 0.95 a_w was about 4 \log_{10} whatever the species.

238

239 **5. Conclusion**

240 Identification of the species and strains responsible for fungal spoilage in dairy industries
241 is important to improve food quality, to control fungal spoilage, but also to optimize
242 disinfection procedures according to the reality of the threat. Moreover, the strain is another
243 important factor to take into account to assess the efficacy of disinfectants, as disinfectant
244 sensitivity variability was observed suggesting that at least few strains should be studied for
245 each considered species.

246 By combining a reduced a_w for the production of conidia and a dry harvesting protocol, a
247 “strong” physiological state, which can be considered as more representative of dairy plant
248 conditions, was obtained. This physiological state can be compared to the “weak”
249 physiological state that results from the preparation of conidial suspensions as recommended
250 by the international standards to assess the efficacy of disinfectants against molds. The impact
251 of the physiological state on the inactivation of conidia could be greater than 1.5 log₁₀, thus
252 suggesting that disinfectants may not be as efficient as expected against conidia that are
253 produced in a dairy environment.

254

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259

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334 **Table 1. List of *Penicillium commune* strains tested and their origin. The strains were provided by the**
335 **University de Bretagne Occidentale Culture Collection (UBOCC).**

Strain	Area	Source
UBOCC-A-112050	French dairy plant	Contaminated blue veined cheese
UBOCC-A-112059	French dairy plant	Contaminated blue veined cheese
UBOCC-A-116003	French dairy plant	Contaminated hard cheese
UBOCC-A-119012	Consumer fridge	Contaminated hard cheese

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337

338

339 **Table 2. Log₁₀ inactivation values (± standard variation) of *Penicillium commune* conidia exposed to 70%**
 340 **ethanol for 1, 2 and 5 minutes.**

Strain	Production of conidia	Harvesting condition	Inactivation value log ₁₀		
			1 min	2 min	5 min
UBOCC-A-112050	0.995 <i>a_w</i>	Hydrated	> 4.06 (± 0.00)	> 4.06 (± 0.00)	> 4.06 (± 0.00)
		Dry-harvested	> 4.46 (± 0.00)	> 4.46 (± 0.00)	> 4.46 (± 0.00)
	0.950 <i>a_w</i>	Hydrated	> 3.85 (± 0.00)	> 3.85 (± 0.00)	> 3.85 (± 0.00)
		Dry-harvested	4.86 (± 0.24)	> 5.12 (± 0.00)	> 5.12 (± 0.00)
UBOCC-A-112059	0.995 <i>a_w</i>	Hydrated	> 4.02 (± 0.00)	> 4.02 (± 0.00)	> 4.02 (± 0.00)
		Dry-harvested	> 4.80 (± 0.00)	> 4.80 (± 0.00)	> 4.80 (± 0.00)
	0.950 <i>a_w</i>	Hydrated	> 4.11 (± 0.00)	> 4.11 (± 0.00)	> 4.11 (± 0.00)
		Dry-harvested	4.62 (± 0.15)	> 5.38 (± 0.00)	> 5.38 (± 0.00)
UBOCC-A-116003	0.995 <i>a_w</i>	Hydrated	> 3.95 (± 0.00)	> 3.95 (± 0.00)	> 3.95 (± 0.00)
		Dry-harvested	> 4.19 (± 0.00)	> 4.19 (± 0.00)	> 4.19 (± 0.00)
	0.950 <i>a_w</i>	Hydrated	> 3.89 (± 0.00)	> 3.89 (± 0.00)	> 3.89 (± 0.00)
		Dry-harvested	2.35 (± 0.17)	3.89 (± 0.26)	> 4.78 (± 0.00)
UBOCC-A-119012	0.995 <i>a_w</i>	Hydrated	> 4.01 (± 0.00)	> 4.01 (± 0.00)	> 4.01 (± 0.00)
		Dry-harvested	> 5.26 (± 0.00)	> 5.26 (± 0.00)	> 5.26 (± 0.00)
	0.950 <i>a_w</i>	Hydrated	> 3.86 (± 0.00)	> 3.86 (± 0.00)	> 3.86 (± 0.00)
		Dry-harvested	5.40 (± 0.00)	> 5.40 (± 0.00)	> 5.40 (± 0.00)

White cells: total inactivation of conidia. Grey cells: presence of visible mycelium after 3 and 7 days at 25 °C.