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

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.

1. Introduction

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

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

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

disinfectant depends greatly on the considered species. For example 0.15% peracetic acid decreased the population of *A. brasiliensis*, *C. albicans* and *P. commune* (CCT 7683) by 0.67, 1.3 and 2.3 $\log_{10}$, respectively (Bernardi et al., 2018). These authors showed that the difference between the species was even more pronounced at 1.5% peracetic acid as these species exhibited decrease of 3 log for *A. brasiliensis* and more than 5 log for *C. albicans* and *P. commune*.

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

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

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

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

2. Materials and methods

2.1. Molds

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

2.2. Conidial production

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

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.

2.5. Viability assessment

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

3. Results

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

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

physiological state were inactivated, inactivation was greater than 3.86, 3.85, and 4.02 $\log_{10}$ for UBOCC-A-119012, UBOCC-A-112050 and UBOCC-A-112059, respectively. The number of conidia obtained for the conditions of the "weak" physiological state group was lower than that the one produced for the "strong" physiological state with the exception of UBOCC-A-112059. Because more conidia were obtained for the "strong" physiological state, inactivation seemed greater than for the "weak" one. In fact, the effect of the physiological state can be accurately determined for the strain UBOCC-A-116003, as all conidia from the "weak" physiological state group were inactivated. Inactivation was greater than 3.89 $\log_{10}$ for this group, whereas the "strong" group exhibited only a 2.35 $\log_{10}$ inactivation value. In this case, the difference between the two groups was greater than 1.54 $\log_{10}$.

The impact of intraspecific variability in *P. commune* could be quantified for the "strong" physiological state only. A group of "sensitive" strains, namely UBOCC-A-112050, UBOCC-A-112059 and UBOCC-A-119012 could be distinguished from the "resistant" UBOCC-A-116003strain. For 1 min treatment, the difference between the inactivation value of the UBOCC-A-116003 and the mean of the 3 "sensitive" strains was 2.61 $\log_{10}$.

4. Discussion

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

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

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

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

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

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

5. Conclusion

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

By combining a reduced a_w for the production of conidia and a dry harvesting protocol, a "strong" physiological state, which can be considered as more representative of dairy plant conditions, was obtained. This physiological state can be compared to the "weak" physiological state that results from the preparation of conidial suspensions as recommended by the international standards to assess the efficacy of disinfectants against molds. The impact of the physiological state on the inactivation of conidia could be greater than 1.5 $\log_{10}$, thus suggesting that disinfectants may not be as efficient as expected against conidia that are produced in a dairy environment.

Acknowledgements

This work was funded by the French Dairy Interbranch Organization (CNIEL) (Moisibio Project, 2019_00621) and the French Association for Research and Technology (ANRT) (CIFRE 2018/0036).

References

Bernardi, A.O., Stefanello, A., Garcia, M.V., Parussolo, G., Stefanello, R.F., Moro, C.B., Copetti, M.V., 2018. Efficacy of commercial sanitizers against fungi of concern in the food industry. LWT 97, 25–30.

Bernardi, A.O., Stefanello, A., Lemos, J.G., Garcia, M.V., Copetti, M.V., 2019. Antifungal activity of commercial sanitizers against strains of *Penicillium roqueforti*, *Penicillium paneum*, *Hyphopichia burtonii*, and *Aspergillus pseudoglaucus*: Bakery spoilage fungi. Food Microbiol. 83, 59–63.

Bundgaard-Nielsen, K., Nielsen, P.V., 1996. Fungicidal effect of 15 disinfectants against 25 fungal contaminants commonly found in bread and cheese manufacturing. J. Food Prot. 59, 268–275.

Burgain, A., Dantigny, P., 2016. Inoculation of airborne conidia of *Penicillium chrysogenum* on the surface of a solid medium. Food Microbiol. 54, 162–166.

Dagnas, S., Membré, J.-M., 2013. Predicting and preventing mold spoilage of food products. J. Food Prot. 76, 538–551.

Dantigny, P., 2016. Relevant issues in predictive mycology. Curr. Opin. Food Sci. 11, 29–33.

Dantigny, P., Nanguy, S.P.-M., 2009. Significance of the physiological state of fungal spores. Int. J. Food Microbiol. 134, 16–20.

Dao, T., Bensoussan, M., Gervais, P., Dantigny, P., 2008. Inactivation of conidia of *Penicillium chrysogenum*, *P. digitatum* and *P. italicum* by ethanol solutions and vapours. Int. J. Food Microbiol. 122, 68–73.

Dao, T., Dantigny, P., 2009. Preparation of fungal conidia impacts their susceptibility to inactivation by ethanol vapours. Int. J. Food Microbiol. 135, 268–273.

European Standard n. 13697, 2001. Chemical disinfectants and antiseptics – Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas - Test method and requirements without mechanical action (phase 2, step 2).

Filtenborg, O., Frisvad, J.C., Thrane, U., 1996. Moulds in food spoilage. Int. J. Food Microbiol. 33, 85–102.

Garnier, L., Valence, F., Pawtowski, A., Auhustsinava-Galerne, L., Frotté, N., Baroncelli, R., Deniel, F., Coton, E., Mounier, J., 2017. Diversity of spoilage fungi associated with various French dairy products. Int. J. Food Microbiol. 241, 191–197.

Heldman, D.R., 1974. Factors influencing air-borne contamination of foods. A review. J. Food Sci. 39, 962–969.

Hocking, A.D., Faedo, M., 1992. Fungi causing thread mould spoilage of vacuum packaged Cheddar cheese during maturation. Int. J. Food Microbiol. 16, 123–130.

Korukluoglu, M., Sahan, Y., Yigit, A., 2006. The fungicidal efficacy of various commercial disinfectants used in the food industry. Ann. Microbiol. 56, 325–330.

Ledenbach, L.H., Marshall, R.T., 2009. Microbiological Spoilage of Dairy Products, in: Sperber, W.H., Doyle, M.P. (Eds.), Compendium of the Microbiological Spoilage of Foods and Beverages. Springer New York, New York, NY, pp. 41–67.

Nesci, A., Etcheverry, M., Magan, N., 2004. Osmotic and matric potential effects on growth, sugar alcohol and sugar accumulation by *Aspergillus* section *Flavi* strains from Argentina. J. Appl. Microbiol. 96, 965–972.

Pitt, J.I., Hocking, A.D., 2009. Introduction, *in*: Fungi and food spoilage. Springer US, Boston, MA, pp. 1–2.

Salo, S., Wirtanen, G., 2005. Disinfectant efficacy on foodborne spoilage yeast strains. Food Bioprod. Process. 83, 288–296.

Salustiano, V.C., Andrade, N.J., Brandão, S.C.C., Azeredo, R.M.C., Lima, S.A.K., 2003. Microbiological air quality of processing areas in a dairy plant as evaluated by the sedimentation technique and a one-stage air sampler. Braz. J. Microbiol. 34, 255–259.

Table 1. List of *Penicillium commune* strains tested and their origin. The strains were provided by the 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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339 **Table 2. Log_{10} inactivation values (± standard variation) of *Penicillium commune* conidia exposed to 70% ethanol for 1, 2 and 5 minutes.**

			Inactivation value log_{10}		
Strain	**Production of conidia**	**Harvesting condition**	**1 min**	**2 min**	**5 min**
UBOCC-A-112050	0.995 a_w	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 a_w	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 a_w	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 a_w	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 a_w	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 a_w	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 a_w	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 a_w	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.