Impact of the physiological state of fungal spores on their inactivation by active chlorine and hydrogen peroxide

Karim Rigalma

▶ **To cite this version:**

Karim Rigalma. Impact of the physiological state of fungal spores on their inactivation by active chlorine and hydrogen peroxide. Food Microbiology, 2021, 100, pp.103850. 10.1016/j.fm.2021.103850 . hal-03736423

HAL Id: hal-03736423
https://hal.inrae.fr/hal-03736423v1

Submitted on 2 Aug 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Version of Record: https://www.sciencedirect.com/science/article/pii/S0740002021001155
Manuscript_96d37dd4ec1ed0e1978482c56f591b76

Impact of the physiological state of fungal spores on their inactivation by active chlorine and hydrogen peroxide

Vincent VISCONTI, Karim RIGALMA, Emmanuel COTON and Philippe DANTIGNY*

Univ Brest, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, F-29280 Plouzané, France

*__Corresponding author__ : Philippe DANTIGNY
Laboratoire Universitaire de Biodiversité et Ecologie Microbienne,
Parvis Blaise-Pascal, Technopôle Brest-Iroise
29280 Plouzané, France
Tel : +33 (0)2.90.91.51.11
E-mail : philippe.dantigny@univ-brest.fr

Keywords: ***Aspergillus***, ***Cladosporium***, ***Mucor***, ***Penicillium***, **fungicide, disinfectant, predictive mycology**

© 2021 published by Elsevier. This manuscript is made available under the CC BY NC user license
https://creativecommons.org/licenses/by-nc/4.0/

Abstract

This study aimed at assessing the impact of the physiological state of fungal spores on inactivation by sodium hypochlorite, 0.1% and 0.2% active chlorine, and 3% hydrogen peroxide. In this context, two physiological states were compared for 4 fungal species (5 strains). The first physiological state corresponded to fungal spores produced at 0.99 a_w and harvested using an aqueous solution (laboratory conditions), while the second one corresponded to fungal spores produced under a moderate water stress (0.95 a_w) and dry-harvested (mechanical harvesting without use of any water, mimicking food plant conditions). *Aspergillus flavus* "food plant" conidia were more resistant to all tested fungicide molecules than the "laboratory" ones. The same phenomenon was observed for *Penicillium commune* UBOCC-A-116003 conidia treated with hydrogen peroxide. However, this isolate did not exhibit any inactivation difference between "laboratory" and "food plant" conidia treated with sodium hypochlorite. Similarly, the physiological state of *Cladosporium cladosporioides* conidia did not impact the efficacy of the tested biocides. *P. commune* UBOCC-A-112059 "food plant" and "laboratory" conidia were more resistant to hydrogen peroxide and sodium hypochlorite, respectively. As for *Mucor circinelloides,* "laboratory" spores were more resistant to all disinfectant than the "food plant" ones. Noteworthy, regardless of the physiological state, all *M. circinelloides* and *C. cladosporioides* conidia were inactivated for 5 min treatment at 0.2% active chlorine and for 2.5 min treatment at 0.1% active chlorine, while the conidia of all the other species remained viable for these treatments. The obtained data indicate that the efficacy of disinfectant molecules depends not only on the encountered fungal species and its intraspecific diversity but also on the spore physiological state.

1. Introduction

Efficacy of fungicide molecules depends on various treatment conditions, i.e., nature of the disinfectant, contact time, concentration, application conditions (temperature, pH, relative humidity, dirtiness of the surfaces, presence of surfactants…) and mode (e.g. fumigation, liquid application) and obviously the targeted fungi. For sanitization efficacy tests, the standard fungal strains are *Aspergillus brasiliensis* (ATCC 16404) and *Candida albicans* (ATCC 10231). According to the European Committee for Standardization, other species can be tested, i.e., *Absidia corymbifera* (IP 1129 75), *Candida albicans* (IP 1180 79), *Cladosporium cladosporioides* (IP 1232 80), *Penicillium verrucosum* var.*cyclopium* (IP 1231 80) to assess fungicidal efficacy (KSG France, 2021). Beyond the fact that the proposed species may not be the most representative of food plant contaminants and, as recently shown by Scaramuzza et al. (2020), might even be more sensitive than the latter; in all cases, fungal spores have to be produced under optimal growth conditions (i.e., temperature, time, atmosphere, medium, (AFNOR NF T 72-281, 2014; European Standard 1275, 2006; European Standard 1650, 2019; European Standard 13624, 2013; European Standard 13697+A1, 2019). These standards recommend Malt Extract Agar (pH 5.6, 0.99 a_w) to produce fungal spores that are subsequently diluted into peptonized water. In contrast to these spores produced in laboratory conditions, in food plant conditions, spores are airborne and not rehydrated, and unlikely to be produced under optimal conditions.

The physiological state of *Penicillium* spp. strains was affected by temperature, water activity and pH during conidia production (Nguyen van Long et al., 2017a). Moreover, the impact of the harvesting protocol on the susceptibility to disinfectants can be important as, for example, wet harvested *Rhizopus stolonifer* conidia had a different physiological state than dry-harvested ones. Even brief exposure (30 min) to water during harvesting caused spores to

engage metabolic activity (Nicherson et al., 1981). The effects of environmental conditions during sporogenesis on the germination time is well documented (Blaszyk et al., 1998; Judet et al., 2008; Nanguy et al., 2010; Nguyen van Long et al., 2017a, 2017b; Lattab et al., 2012; Ruijten et al., 2020; Stevenson et al., 2016). Less information on the impact of environmental conditions on the susceptibility to disinfectants is available. Dry-harvested *Penicillium* conidia exposed to ethanol vapours for 24h did not show any inactivation whereas aqueous suspensions of *P. digitatum* and *P. italicum* conidia exhibited about 3 log and 2 log inactivation, respectively (Dao and Dantigny, 2009). The same authors showed that the contact time with ethanol vapours (0.67 kPa) required to inactivate 4 log *P. chrysogenum* and *P. digitatum* conidia produced at either 0.99 a_w or in the 0.85-0.90 a_w range were about 30h and 120h, respectively (Dao and Dantigny, 2009).

Production of conidia at reduced water activity increases the amount of protective compatible solutes in the cytoplasm (Andersen et al., 2006; Chandler et al., 1984; de Lima Alves et al., 2015; Hallsworth and Magan, 1995; Magan, 2006, 2007; Nesci et al., 2014; Rangel et al., 2015; Wyatt et al., 2013, 2015) that can contribute to a certain resistance. It was also hypothesized that both the production of conidia at reduced water activity and application of a dry-harvesting protocol contributed to lower the intracellular water activity of fungal spores (Dao and Dantigny, 2009), thus explaining their resistance to ethanol which is an hydrophilic molecule. This hypothesis was strengthen by the observation that for 1 min exposure to 70% ethanol, only dry-harvested conidia of four *Penicillium commune* strains produced at 0.95 a_w exhibited survivors, while suspensions of conidia produced at 0.995 a_w were completely inactivated (Visconti et al. 2020).

This study aimed at extending the comparison between “laboratory” conidia (production at 0.995 a_w and harvested using an aqueous solution) and “food plant” (production at 0.95 a_w and dry-harvested) conidia to other fungal species and disinfectant molecules. The ethanol

93 resistant *P. commune* UBOCC-A-116003 and the ethanol sensitive *P. commune* UBOCC-A-
94 112059 (Visconti et al. 2020) were selected, in addition to species that are frequently isolated
95 from dairy product environments, namely *Cladosporium cladosporioides*, *Mucor*
96 *circinelloides* and *Aspergillus flavus*. The tested disinfectant molecules corresponded to
97 sodium hypochlorite (0.1 and 0.2% active chlorine) and hydrogen peroxide (3%).

98

99 **2. Material and methods**

100 *2.1. Molds*

101 The studied molds were provided by the Université de Bretagne Occidentale Culture
102 Collection (UBOCC, https://www.univ-brest.fr/ubocc/) and were originally isolated from
103 spoiled dairy products (Table 1). Molds were maintained on Potato Dextrose Agar (PDA)
104 medium at 4°C.

105

106 *2.2. Spores production*

107 "Laboratory" spores were produced from mycelium grown on PDA at 0.995 a_w for 7 days at
108 25°C and harvested in sterile saline solution (NaCl, 9 g/l of water) containing Tween 80
109 (0.015% v/v).
110 "Food plant" spores were produced from mycelium grown on PDA at 0.950 a_w for 7 days at
111 25°C. Water activity was adjusted by substituting a part of the water with an equal weight of
112 glycerol. The relative amount of glycerol was 20.6% (w/w) to obtain PDA media at 0.950 a_w.
113 Spores were harvested mechanically without contact with any liquid solution (dry-harvest)
114 according to Dao and Dantigny (2009). Briefly, ten sterile glass beads (diameter 3 mm) were
115 deposited on the mycelium and the Petri dish was shaken gently to detach the conidia. Then,
116 the plate was turned upside-down and the conidia were harvested on the lid by gently tapping
117 100 times on the bottom of the Petri dish.

2.3. Disinfectants

Sodium hypochlorite test solutions were prepared from a stock solution diluted in distilled water containing Tween 80 (0.015%, v/v). The stock solution was prepared from 250 ml bleach boxes (Eau de Javel La Croix, Bois-Colombes, France) that contained 4.8% active chlorine (5% hypochlorite), diluted with distilled water according to the supplier's recommendation to reach final concentrations of 0.1 and 0.2% active chlorine. The co-formulants mentioned by the supplier were 5-15% chlorine bleach and sodium hydroxide. The hydrogen peroxide test solution was prepared from 30% hydrogen peroxide solution (Sigma Aldrich, Saint-Quentin Fallavier, France) diluted in distilled water containing Tween 80 (0.015%, v/v) to reach a 3% final concentration.

2.4. Disinfectant testing procedures

Treatment of "food plant" spores started by applying 10 ml of biocide solution to the dry-conidia on the lid. For *M. circinelloides*, this volume was reduced to 5 ml as fewer spores were harvested. Prior to the first sampling, the suspension (biocide + conidia) was transferred to a Falcon tube and homogenized by vortexing for 10 s. Treatment of "laboratory" spores began when 1ml of spore suspension was transferred in 9 ml of biocide solution and the mix vortexed. For sodium hypochlorite, spores survival was measured every 2.5 min for 20 min except for *M. circinelloides* for which an additional contact time of 1 min was done. For hydrogen peroxide, spores survival was measured after 1, 5, 10, 15, 30 and 60 min of contact time. Both biocides were neutralized by diluting ten-fold the suspension in 1% sodium thiosulfate pentahydrate diluted in saline aqueous solution (NaCl, 9 g/l) containing Tween 80 (0.015% v/v). The initial amount of "food plant" spores (N_0) was evaluated by counting on a Malassez cell according to Dao et al. (2008). The method was based on the principle that the

application of biocide does not affect the morphology of the spores during the time of the experiment so it is possible to count under the microscope all the spores treated including inactivated ones.

2.5. Viability assessment

After neutralization, the treated spores were grown on PDA at 25 °C for 3 days by spreading 100 µl of sample and four subsequent decimal dilutions. *M. circinelloides* spores were grown only for 1 day. Only counts (N) in the range of 10 to 100 colonies per plate were considered. Inactivation of conidia was expressed as the logarithmic reduction factor, $\log_{10}(N/N_0)$.

3. Results

3.1. Impact of disinfectant molecules on "laboratory" spores

At 0.1% active chlorine, *C. cladosporioides* was the most sensitive species (Fig. 1A), as for a contact time of 2.5 min, *C. cladosporioides* showed 2.8 log inactivation. Inactivation were less than or equal to 0.2 log for all the other tested species. At 12.5 min of exposure, *P. commune* only exhibited survivors, the UBOCC-A-112059 isolate was more resistant than the UBOCC-A-116003.

For a contact time of 2.5 min to 0.2% active chlorine, total inactivation of *C. cladosporioides* and *M. circinelloides* spores was observed, (Fig. 1B). At 7.5 min of exposure, *P. commune* only exhibited survivors. Inactivation values were 1.9 log and 3.1 log for the UBOCC-A-112059 and UBOCC-A-116003 strains, respectively. Under these experimental conditions, all *A. flavus* conidia were inactivated.

By increasing the active chlorine concentration from 0.1 to 0.2%, the contact time leading to a complete inactivation of the conidia was reduced from 5 to 2.5 min and, from 15 to 10 min,

for *C. cladosporioides* and *P. commune*, respectively. Regardless of the tested concentration, the former and the latter species were the most sensitive and most resistant to active chlorine, respectively.

In contrast to active chlorine, *P. commune* was the most sensitive species to 3% hydrogen peroxide (Fig. 1C). For a 10 min contact time, all spores of the two tested strains were inactivated. After 30 min of exposure, all *A. flavus* spores were inactivated, inactivation being greater than 4 log. In contrast to this species, inactivation was only 0.2 log for *C. cladosporioides* and *M. circinelloides*, which were the most resistant species.

3.2. Impact of fungicide molecules on "food plant" spores

As for the physiological "food plant" state, *M. circinelloides* was the most susceptible mold to 0.1% active chlorine. At 2.5 min of exposure, all *M. circinelloides* spores were inactivated, inactivation being greater than 4.3 log (Fig. 2A). For this contact time, the inactivation of *C. cladosporioides* was equal to 2.7 log, and less than or equal to 0.2 log for *A. flavus* and *P. commune*. For the “food plant” physiological state, *A. flavus* was the most resistant species, the only one to exhibit survivors for a contact time of 17.5 min.

By increasing the active chlorine concentration, the contact time necessary to inactivate all the spores was reduced. After 2.5 min of exposure, all *C. cladosporioides* conidia and *M. circinelloides* spores were inactivated, while inactivation was lower than or equal to 0.4 log for *A. flavus* and *P. commune* (Fig. 2B). At 0.2% active chlorine, *P. commune* UBOCC-A-116003 was more resistant than the UBOCC-A-112059 strain. No *A. flavus* survivors were observed for a contact time of 12.5 min.

A. flavus was the most sensitive species to 3% hydrogen peroxide. A complete inactivation was observed for a contact time of 30 min (Fig. 2C). After 60 min exposure, only *C.*

191 *cladosporioides* (1.2 log inactivation) and *P. commune* UBOCC-A-112059 (2.4 log
192 inactivation) exhibited survivors. All spores of *M. circinelloides* and *P. commune* UBOCC-A-
193 116003 were inactivated (> 4 and > 4.4 log, respectively).

194

195 *3.3. Impact of the physiological state on the disinfectant efficacy*

196 In order to assess the impact of the physiological state on the efficacy of the fungicide
197 molecules, inactivation were compared at the same time, although this time depended on the
198 considered molecule and species. Whenever possible, the shortest contact time leading to no
199 survivors, for either “laboratory” or “food plant” spores, was chosen (Table 2). Four different
200 cases were distinguished, i)”laboratory” spores were more resistant than “food plant” ones, ii)
201 no significant differences were observed between the two physiological states, iii)
202 impossibility to determine whether the two kind of spores exhibited significant differences
203 and iv)”food plant” spores were more resistant than “laboratory” ones.

204 For *M. circirnelloides*, the "laboratory" spores were more resistant than the "food plant"
205 spores to two disinfectants. For a contact time of 2.5 min at 0.1% active chlorine, inactivation
206 was 0.1 log and greater than 4.3 log for the "laboratory" and "food plant" spores, respectively.
207 The difference in inactivation between the two physiological states was greater than 4.2 log.
208 After exposure to 0.2% active chlorine for 1 min, inactivation was 0.2 log and greater than 4.3
209 log for "laboratory" and "food plant" spores, respectively. The difference in inactivation
210 between the two physiological states was greater than 4.1 log. For *P. commune* UBOCC-A-
211 112059, the "laboratory" conidia were more resistant than the "food plant" ones to active
212 chlorine. For a contact time of 12.5 min at 0.1% active chlorine, inactivation was 1.5 log and
213 3 log for the "laboratory" and "food plant" conidia, respectively. The difference in
214 inactivation between the two physiological states was 1.5 log. At 0.2% active chlorine for 1

min, inactivation was 1.9 and 4.6 log for "laboratory" and "food plant" conidia, respectively, the difference in inactivation being 2.7 log between the two physiological states.

No significant impact was observed between the inactivation by sodium hypochlorite of the two physiological states of *P. commune* UBOCC-A-116003. For a contact time of 12.5 min at 0.1% active chlorine, inactivation was 3 and 3.1 log for the "laboratory" and "food plant" spores, respectively. For a contact time of 7.5 min at 0.2% active chlorine, inactivation was 3.1 and 3.5 log for "laboratory" and "food plant" physiological states, respectively. For *C. cladosporioides*, no significant difference was observed between the two physiological states at 0.1% active chlorine and 3% hydrogen peroxide. Indeed, at 0.1% active chlorine for 2.5 min, the inactivation was 2.7 and 2.8 log for the "food plant" and "laboratory" spores, respectively, while, at 3% hydrogen peroxide for 60 min, inactivation was 1.2 and 1.4 log for the same physiological states, respectively.

As for the impact of physiological state after exposure to 0.2% active chlorine, it was impossible to determine as all conidia were inactivated from 2.5 min (inactivation > than 4.1 log).

For *A. flavus*, the “food plant” conidia were more resistant than the “laboratory” spores for the two tested disinfectants. For a contact time of 12.5 min at 0.1% active chlorine, inactivation was 0.7 log and greater than 3.7 log for the "food plant" and "laboratory" conidia, respectively, difference in inactivation between the two physiological states being greater than 3 log. For 0.2% active chlorine for 7.5 min, inactivation was 0.9 log and greater than 4.3 log for the "food plant" and "laboratory" conidia, respectively, and the difference in inactivation between the two physiological states was greater than 3.4 log. For a contact time of 15 min with 3% hydrogen peroxide, inactivation was 1.4 log and 3.6 log for the "food plant" and "laboratory" conidia, respectively. The difference in inactivation between the two physiological states was 2.2 log. Finally, for 3% hydrogen peroxide, the two *P. commune*

"food plant" conidia were more resistant than the "laboratory" ones. At a contact time of 10 min, inactivation of *P. commune* UBOCC-A-112059 was nil and greater than 4.1 log for the "food plant" and "laboratory" conidia, respectively. The difference in inactivation between the two physiological states was greater than 4.1 log. For the same contact time, inactivation of *P. commune* UBOCC-A-116003 was 0.7 log and greater than 4.2 log for the "food plant" and "laboratory" conidia, respectively (difference in inactivation between the two physiological states being greater than 3.5 log).

3.4. Efficacy of fungicide molecules on the species

With the exception of *C. cladosporioides* and *M. circinelloides*, a contact time of 5 min to 0.1% active chlorine or 2.5 min to 0.2% active chlorine had almost no effect on the tested species. However, inactivation obtained for a contact time of 7.5 min to 0.2% active chlorine were of the same order of magnitude than treatments to 0.1% active chlorine for 12.5 min, except for “food plant” *P. commune* UBOCC-A-112059 conidia. However, these conditions were not sufficient to completely inactivate the two *P. commune* strains and the *A. flavus* “food plant” conidia. These conidia were the most resistant ones to sodium hypochlorite.

A contact time of 15 min to 3% hydrogen peroxide did not inactivate the spores of the tested species, except the “laboratory” *P. commune* conidia. In contrast to sodium hypochlorite, the *A. flavus* “food plant” conidia were not the most resistant ones to hydrogen peroxide. Surprisingly, *C. cladosporioides* and *M. circinelloides* exhibited a stronger resistance than the other species to a 15 min treatment to 3% hydrogen peroxide. Overall, at the respective tested concentrations, a longer time was required to inactivate the mold species with hydrogen peroxide than with sodium hypochlorite.

4. Discussion

In order to assess a fungicidal efficacy, many studies examined the effect of disinfectant molecules for many contact times, but very few studies described inactivation kinetics. For chlorine inactivation, a classical linear decrease of the log survivors *versus* time with (for *Aspergillus fumigatus*) or without (for *Aspergillus versicolor* and *Penicillium purpurogenum*) an initial lag phase was observed (Ma and Bibby, 2017). In our study, a clear presence of an initial lag phase was shown for the inactivation of "food plant" *P. commune* conidia with 0.1% active chlorine. Due to very fast inactivation kinetics leading to very few experimental data, our results did not show a clear linear decrease in the log survivors. However, inactivation curves exhibiting a tailing off (Pereira et al., 2013; Salomão et al., 2009) were not observed in our study. Inactivation kinetics in drinking water were evaluated by means of Ct ($mg.min.l^{-1}$), i.e., concentration x time (Ma and Bibby, 2017, Pereira et al., 2013), these approaches assumed an inactivation rate proportional to the disinfectant concentration. For a given Ct value, inactivation should be the same regardless of the time and the concentration. In our study, inactivation for 0.1% active chlorine x 5 min and 0.2% active chlorine x 2.5 min were not significantly different regardless of the considered species or physiological state. Although this approach was not validated for other concentration x time conditions or other disinfectants.

Although the concept of dry-harvested spores was introduced as early as 1981 by Nickerson et al., almost all publications concern spores produced under optimal conditions and suspended into aqueous solutions (designated as "laboratory" spores in this study). In this context, Andrews (1986) found that 0.37% hypochlorite efficiently killed *A. flavus* after a 2 min contact time. In another study, seven *A. flavus* isolates conidia were treated with a diluted commercial disinfectant for 72h. Five isolates did not show any survivors, i.e., log reduction greater than or equal to 2.7, for sodium hypochlorite concentrations in the range 0.4-1.6%.

However, the two other isolates exhibited survivors at 1.6% (Mattei et al., 2013). Eventually, 4.3 log reduction for *A. flavus* conidia exposed to 3% hypochlorite (2.88% active chlorine) for 10 min was reported by Bundgaard-Nielsen and Nielsen (1995). In our study, no survivors were detected after 10 min treatment to 0.2% active chlorine, thus suggesting that the *A. flavus* isolate tested in our study was more sensitive than that of the other study. This is a potential indication of behaviour heterogeneity within a given species.

The effect of sodium hypochlorite (0.1, 0.5 and 1%), was tested against *C. cladosporioides* for 15 min (Bernardi et al., 2018) exhibiting log reductions greater than 4. In our study no survivors (log reduction greater than 3.9) were detected for 5 min treatment at 0.1%. As Bernardi et al. (2018) did not carry out treatment at contact times different than 15 min, it is therefore difficult to compare. Noteworthy, these authors also tested *P. commune* conidia that exhibited 2-2.9 log reduction and more than 4 log reduction at 0.1% and 0.5/1% active chlorine, respectively. Our results showed 1.5 and 3 log reductions depending on the considered *P. commune* strain for 12.5 min treatments at 0.1% active chlorine. Therefore, the obtained results in this study are in accordance with those of Bernardi et al. (2018) despite slightly different contact times and methods (i.e., carrier method in Bernardi et al. (2018) and suspension method in this study). However, as previously mentioned for *A. flavus*, intraspecific diversity may also be an important factor to explain contrasted inactivation results, as 10 min treatments at 3% hypochlorite led to log inactivation in the range 1.7->5.2 for other *P. commune* isolates (Bundgaard-Nielsen and Nielsen, 1995). In agreement with disinfectant susceptibility heterogeneity within a considered species, these authors also reported great differences of behaviour of five *Penicillium roqueforti* isolates when exposed to 0.3% hypochlorite for 10 min (log reductions ranged from 0.5 for the most resistant strain to 4.5 for the most sensitive one, while for the other strains, reductions were in the 1-2 log range).

For 15 min treatments with hydrogen peroxide (at 20°C), 3 log reduction in viable counts were obtained for 12.5%, *Absidia corymbifera*, 3.9%, *Candida albicans*, 5.51%, *Scopulariopsis brevicaulis*, 1.71%, *Aspergillus versicolor* and *Penicillium cyclopium*, and 0.39%, *Geotrichum candidum* (Martin and Maris, 2012). In this study, 3 log and 3.7 log reduction of *A. flavus* conidia were obtained for 10 min and 15 min treatments with 3% hydrogen peroxide, respectively. For this species, a hydrogen peroxide concentration close to 3% would have been required to obtain 3 log reduction in 15 min. According to our results, concentrations lower than (for both *P. commune* strains) and greater than (for *C. cladosporioides* and *M. circinelloides*) than 3% would have been necessary to obtain the same reduction.

In contrast to peroxide hydrogen, *P. commune* was more resistant than *C. cladosporioides* to active chlorine. This result is in accordance with the results reported by Bernardi et al. (2018) indicating, for 15 min treatments at 0.1% sodium hypochlorite, 2-2.9 log and more than 4 log reduction in *P. commune* and *C. cladosporioides* viable conidia, respectively. In accordance with Bundgaard-Nielsen and Nielsen (1995), *P. commune* was more resistant than *A. flavus* to active chlorine. Overall, both *P. commune* strains were more resistant to active chlorine than *A. flavus*, followed by *C. cladosporioides* and *M. circinelloides*. Surprisingly, the rank of resistance was the reverse than the one observed for hydrogen peroxide, *C. cladosporioides* > *M. circinelloides* > *A. flavus* > *P. commune*.

Noteworthy, the resistance ranking differed slightly for “food plant” spores. *A. flavus* was the more resistant and the more sensitive species to active chlorine and hydrogen peroxide, respectively. The rank of resistance depended also on the considered strain; indeed, whereas *P. commune* UBOCC-A-116003 was more resistant than the UBOCC-A-112059 to 70% ethanol (Visconti et al., 2020), UBOCC-A-112059 was more resistant than UBOCC-A-116003 to 3% hydrogen peroxide. This study also showed that differences in resistance

depended on the concentration of the disinfectant, at 0.1% active chlorine no difference was noticed between the two *P. commune* strains, while, at 0.2% active chlorine, UBOCC-A-116003 was more resistant than UBOCC-A-112059. At the tested concentrations in this study, active chlorine was more efficient than hydrogen peroxide. In this study, *P. commune* UBOCC-A-112059 "laboratory" conidia were more resistant than the other isolate to active chlorine, but less resistant than the other isolate to hydrogen peroxide. The impact of the physiological state was greater than 1.54 log inactivation for *P. commune* UBOCC-A-116003 treated with 70% ethanol (Visconti et al., 2020), but the impact was even greater than 3.5 log for 10 min contact time to 3% peroxide hydrogen. "Food plant" *A. flavus* conidia were also more resistant than "laboratory" ones, but *M. circinelloides* "laboratory" spores were more resistant to all disinfectants than the "food plant" ones. In contrast to the other species, *M. circinelloides* can hardly develop at water activities below 0.90 a_w (Hocking and Miscamble, 1995; Tresner and Hayes, 1971) and at 0.95 a_w, the growth rate was shown to about half the optimum growth rate (Morin-Sardin et al., 2016). In contrast, *A. flavus* is a xerophilic species that can grow at water activities less than 0.85 a_w, (Pitt and Miscamble, 1995; Sautour et al., 2001, 2002). At 0.95 a_w the growth rate was almost the same than that observed at the optimum water activity of 0.974 (Sautour et al., 2001). These observations suggested that the increased sensitivity to disinfectants of *M. circinelloides* "food plant" spores may be due to the fact that these spores were produced at a water activity (0.95 a_w) too close to the minimum one.

5. Conclusions

Surface disinfection in food industries should be based on the use of most effective sanitizer, for the best "contact time / concentration" combination and taking into account the most resistant mold, in agreement with a "worst case scenario" approach. In many cases, a lag

364 phase prior to inactivation was observed. Therefore, shortening the contact time could lead to
365 the elimination of the disinfectant even before fungal spores are actually killed. What can be
366 considered as the most resistant mold depends not only on the encountered species and strains
367 within a given species (disinfectant susceptibility heterogeneity), but also, as demonstrated in
368 this study, on the physiological state of fungal spores and even on the considered disinfectant
369 molecule. Based on the obtained data, it is recommended to assess fungicidal activity of
370 disinfectants on "food plant" spores, not only because there were more resistant than
371 "laboratory" ones for the most resistant species, but because the "food plant" physiological
372 state was closer to airborne conidia that are responsible for actual food contamination and
373 spoilage.

374

375 **Acknowledgements**

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

379

References

Agence Française de Normalisation NF T 72-281, 2014. Procédés de désinfection des surfaces par voie aérienne - Détermination de l'activité bactéricide, fongicide, levuricide, mycobactéricide, tuberculocide sporicide et virucide incluant les bactériophages.

Andersen, M., Magan, N., Mead, A., Chandler, D., 2006. Development of a population based threshold model of conidial germination for analysing the effects of physiological manipulation on the stress tolerance and infectivity of insect pathogenic fungi. Environ. Microbiol. 8, 1625–1634.

Andrews, S., 1986. Optimisation of conditions for the surface disinfection of sorghum and sultanas using sodium hypochlorite solutions. *In*: A.D. King. J.I. Pitt, L.R. Beuchat and J.E.L. Corry (editors), Methods for the Mycological Examination of Food. Plenum Press, New York. pp. 28-32.

Andrews, S., 1996. Evaluation of surface disinfection procedures for enumerating fungi in foods: a collaborative study. Int. J. Food Microbiol. 29, 177-184.

Andrews, S., Pardoel, D., Harun, A., Treloar, T., 1997. Chlorine inactivation of fungal spores on cereal grains. Int. J. Food Microbiol. 35, 153-162.

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 – Food Sci. Technol. 97, 25-30.

403 Blaszyk, M., Blank, G., Holley, R., Chong, J., 1998. Reduced water activity during
404 sporogenesis in selected *Penicillia*: impact on spore quality. Food Res. Int. 31, 503-509.

405 Bundgaard-Nielsen, K. and Nielsen, P.V., 1995. Fungicidal effect of 15 disinfectants against
406 25 fungal contaminants commonly found in bread and cheese manufacturing. J. Food
407 Prot. 59, 268-275.

408 Chandler, D., Crowe, L.M., Chapman, D., 1984. A population based threshold model to
409 demonstrate that physiological manipulation of endogenous reserves can increase the
410 virulence of insect pathogenic fungi. BCPC Crop Science and Technology 4B, 309–314.

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

413 de Lima Alves, F., Stevenson, A., Baxter, E., Gillion, J.L.M., Hejazi, F., Hayes, S., Morrison,
414 I.E.G., Prior, B.A., McGenity, T.J., Rangel, D.E.N., Magan, N., Timmis, K.N.,
415 Hallsworth, J.E., 2015. Concomitant osmotic and chaotropicity-induced stresses in
416 *Aspergillus wentii*: compatible solutes determine the biotic window. Curr. Genet. 61,
417 457–477.

418 European Standard n. 1275, 2006. Chemical disinfectants and antiseptics Quantitative
419 suspension test for the evaluation of basic fungicidal or basic yeasticidal activity of
420 chemical disinfectants and antiseptics - Test method and requirements (Phase 1)

421 European Standard n. 13624, 2013. Chemical disinfectants and antiseptics - Quantitative
422 suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area
423 - Test method and requirements (phase 2, step 1)

424 European Standard n. 13697+A1, 2019. Chemical Disinfectants and Antiseptics –
425 Quantitative Non-porous Surface Test for the Evaluation of Bactericidal and/or

426 Fungicidal Activity of Chemical Disinfectants Used in Food, Industrial, Domestic and
427 Institutional Areas –Test Method and Requirements Without Mechanical Action (Phase
428 2, Step 2).

429 European Standard n. 1650, 2019. Chemical disinfectants and antiseptics - Quantitative
430 suspension test for the evaluation of fungicidal or yeasticidal activity of chemical
431 disinfectants and antiseptics used in food, industrial, domestic and institutional areas -
432 Test method and requirements (Phase 2, step 1)

433 Hallsworth, J.E., Magan, N., 1995. Manipulation of intracellular glycerol and erythritol
434 enhances germination of conidia at low water availability. Microbiology 141, 1109–
435 1115.

436 Hocking, A.D. and Miscamble, B.F., 1995. Water relations of some Zygomycetes isolated
437 from food. Mycol. Res. 99, 1113-1119.

438 Judet, D., Bensoussan, M., Perrier-Cornet, J.-M., Dantigny, P., 2008. Distributions of the
439 growth rate of the germ tubes and germination time of *Penicillium chrysogenum* conidia
440 depend on water activity. Food Microbiol. 25, 902-907.

441 KSG France, 2021. Les Normes Afnor ou EN, les méthodes, les marquages et les étiquettes.
442 https://www.ksg-france.fr/les-normes-afnor-ou-en-les-methodes-les-marquages-et-les-
443 etiquettes/ Accessed date: 26 March 2021.

444 Lattab, N., Kalai, S., Bensoussan, M., Dantigny, P., 2012. Effect of storage conditions
445 (relative humidity, duration, and temperature) on the germination time of *Aspergillus*
446 *carbonarius* and *Penicillium chrysogenum*. Int. J. Food Microbiol. 160, 80–84.

447 Ma, X. and Bibby, K., 2017. Free chlorine and monochloramine inactivation kinetics of
448 *Aspergillus* and *Penicillium* in drinking water. Water Res. 120, 265-271.

449 Magan, N., 2006. Ecophysiology of biocontrol agents for improved competence in the
450 phyllosphere. *In*: Bailey, M.J., Lilley, A.K., Timms-Wilson, T.M., Spenser-Phillips,
451 P.T.N. (Eds.), Microbial Ecology of Aerial Plant Surfaces. In CABI International, 149-
452 164.

453 Magan, N., 2007. Fungi in extreme environments. In Environmental and Microbial
454 Relationships, In: Kubicek, C.P., Druzhinina, I.S. (Eds.), The Mycota IV, 2nd edn.
455 Springer-Verlag, Berlin, Germany, pp. 85–103.

456 Martin, H. and Maris, P., 2012. Synergism between hydrogen peroxide and seventeen acids
457 against five agri-food-borne fungi and one yeast strain. J. Appl. Microbiol. 113, 1451-
458 1460.

459 Mattei, A.S., Madrid, I.M., Santin, R., Schuch, L.F.D., Meireles, M.C.A., 2013. *In vitro*
460 activity of disinfectants against *Aspergillus* spp. Braz. J. Microbiol. 44, 481-484.

461 Morin-Sardin, S., Rigalma, K., Coroller, L., Jany, J-L., Coton, E., 2016. Effect of
462 temperature, pH, and water activity on *Mucor* spp. growth on synthetic medium, cheese
463 analog and cheese. Food Microbiol. 56, 69-79.

464 Nanguy, S.P.-M., Perrier-Cornet, J.-M., Bensoussan, M., Dantigny, P., 2010. Impact of water
465 activity of diverse media on spore germination of *Aspergillus* and *Penicillium* species.
466 Int. J. Food Microbiol. 142, 273-276.

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

470 Nguyen Van Long, N., Rigalma, K., Coroller, L., Dadure, R., Debaets, S., Mounier, J.,
471 Vasseur, V., 2017b. Modelling the effect of water activity reduction by sodium chloride

or glycerol on conidial germination and radial growth of filamentous fungi encountered in dairy foods. Food Microbiol. 68, 7-15.

Nguyen Van Long, N., Vasseur, V., Coroller, L., Dantigny, P., Le Panse, S., Weill, A., Mounier, J., Rigalma, K., 2017a. Temperature, water activity and pH during conidia production affect the physiological state and germination time of *Penicillium* species. Int. J. Food Microbiol. 241, 151-160.

Nickerson, K.W., Freer, S.N., Etten, J.L.V., 1981. *Rhizopus stolonifer* sporangiospores: a wet-harvested spore is not a native spore. Exp. Mycol. 5, 189-192.

Pitt, J.I. and Miscamble, B.F., 1995. Water relations of *Aspergillus flavus* and related species. J. Food Prot. 58, 86-90.

Rangel, D.E.N., Braga, G.U.L., Fernandes, É.K.K., Keyser, C.A., Hallsworth, J.E., Roberts, D.W., 2015. Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during conidial formation. Curr. Genet. 61, 383–404.

Ruijten, P., Huinink, H.P., Adan, O.C.G., 2020. *Penicillium rubens* germination on desiccated and nutrient-depleted conditions depends on the water activity during sporogenesis.

Salamão, B.C.M., Churey, J.J., Aragão, G.M.F., Worobo, R.W., 2009. Modeling *Penicillium expansum* resistance to thermal and chlorine treatments. J. Food Microbiol. 72, 2618-2622.

Scaramuzza, N., Mutti, P., Cigarini, M., Berni, E., 2020. Effect of peracetic acid on ascospore-forming molds and test microorganisms used for bio-validations of sanitizing processes in food plants. Int. J. Food Microbiol. 332:108772

493 Sautour, M., Dantigny, P., Divies, C., Bensoussan, M., 2001. A temperature-type model for
494 describing the relationship between fungal growth and water activity. Int. J. Food
495 Microbiol. 67, 63-69.

496 Sautour, M., Soares Mansur C., Divies, C., Bensoussan, M., Dantigny, P., 2001. Comparison
497 of the effects of temperature and water activity on growth rate of food spoilage moulds.
498 J. Ind. Microbiol. Biotechnol. 28, 311-315.

499 Stevenson, A., Hamill, P.G., Medina, A., Kminek, G., Rummel, J.D., Dijksterhuis, J., Timson,
500 D.J., Magan, N., Leong, S.-L.L., Hallsworth, J.E., 2016. Glycerol enhances fungal
501 germination at the water-activity limit for life. Environ. Microbiol. 19, 947-967.

502 Tresner, H.D. and Hayes, J.A., 1971. Sodium chloride tolerance of terrestrial fungi. Appl.
503 Microbiol. 22, 210-213.

504 Visconti, V., Rigalma, K., Coton, E., Dantigny, P., 2020. Impact of intraspecific variability
505 and physiological state on *Penicillium commune* inactivation by 70% ethanol. Int. J.
506 Food Microbiol. 332, 108782.

507 Wyatt, T.T., Golovina, E.A., van Leeuwen, M.R., Hallsworth, J.E., Wösten, H.A.B.,
508 Dijksterhuis, J., 2015. A decrease in bulk water and mannitol and accumulation of
509 trehalose and trehalose-based oligosaccharides define a two-stage maturation process
510 towards extreme stress resistance in ascospores of *Neosartorya fischeri* (*Aspergillus*
511 *fischeri*). Environ. Microbiol. 17, 383–394.

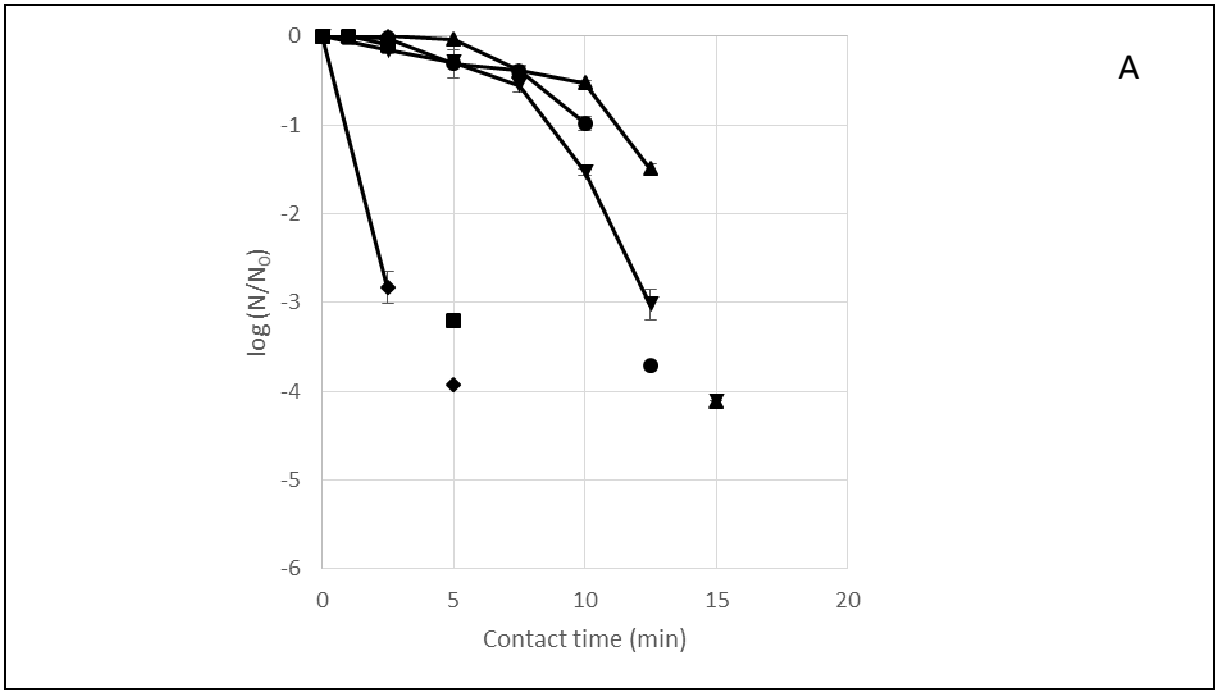
512 Wyatt, T.T., Wösten, H.A.B., Dijksterhuis, J., 2013. Fungal spores for dispersion in space and
513 time. Adv. Appl. Microbiol. 85, 43–91.

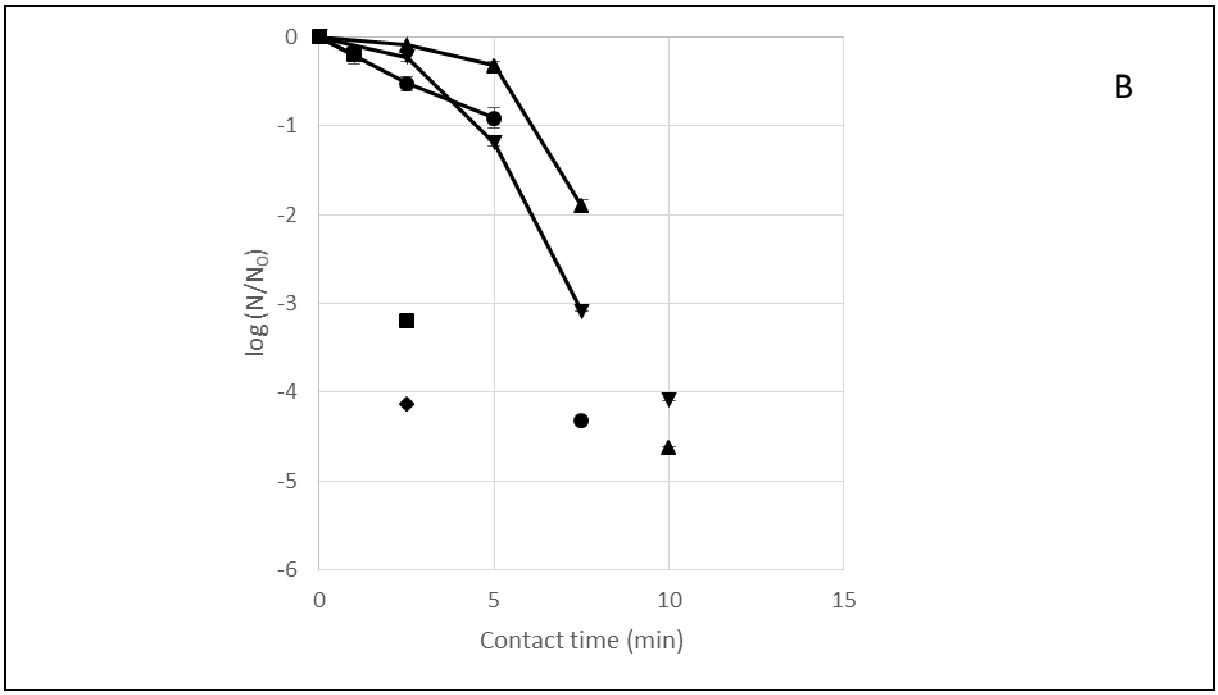
514

Figure legends

Fig. 1. Inactivation of "laboratory" spores (produced at 0.99 a_w and harvested using an aqueous solution) of *Aspergillus flavus* (●), *Cladosporium cladosporioides* (◆), *Mucor circinelloides* (■), *Penicillium commune* (UBOCC-A-112059) (▲) and *Penicillum commune* (UBOCC-A-116003) (▼). Treatment with A) 0.1% active chlorine, B) 0.2% active chlorine and C) 3% hydrogen peroxide. Linked symbols = presence of survivors and isolated symbol = absence of survivors. Error bars represent standard deviations.

Fig. 2. Inactivation of "food plant" spores (produced at 0.95 a_w and dry-harvested) of *Aspergillus flavus* (○), *Cladosporium cladosporioides* (◇), *Mucor circinelloides* (□), *Penicillium commune* (UBOCC-A-112059) (△) and *Penicillum commune* (UBOCC-A-116003) (▽). Treatment with A) 0.1% active chlorine, B) 0.2% active chlorine and C) 3% hydrogen peroxide. Linked symbols = presence of survivors and isolated symbol = absence of survivors. Error bars represent standard deviations.


A
0
-1
-2
-3
-4
-5
-6
log (N/N0)
0
5
10
15
20
Contact time (min)


B
0
-1
-2
-3
-4
-5
-6
log (N/N0)
0
5
10
15
Contact time (min)

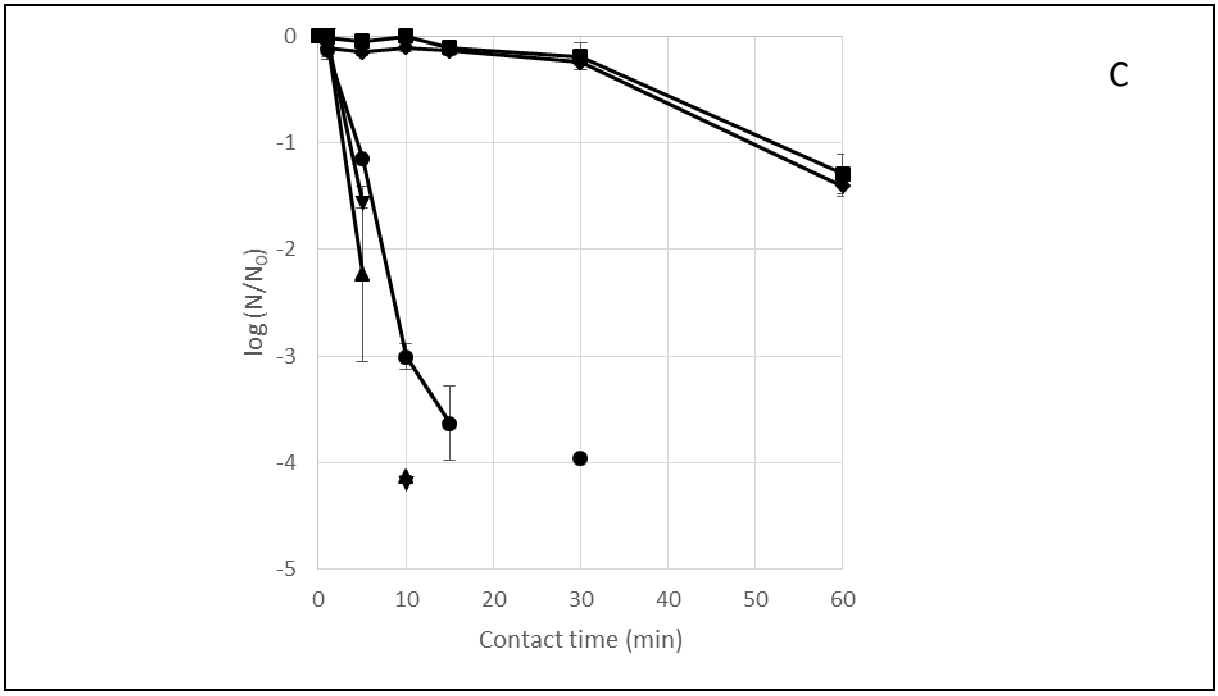
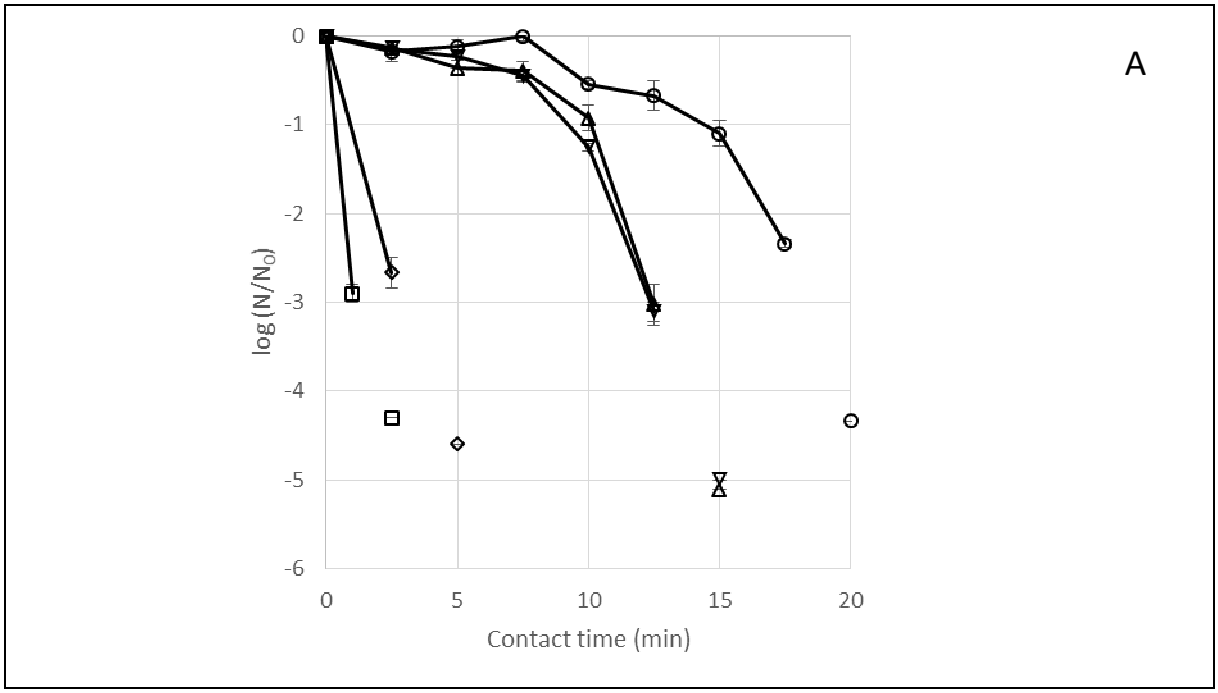
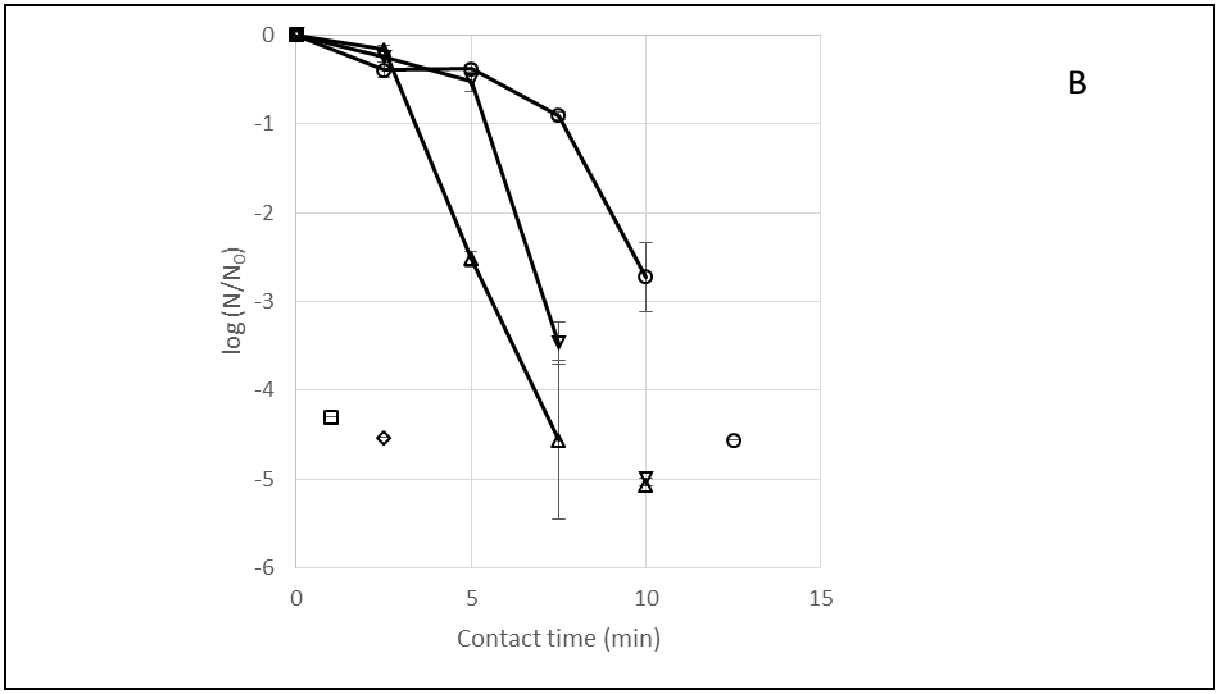


Figure 1


A
0
-1
-2
-3
-4
-5
-6
log (N/N0)
0
5
10
15
20
Contact time (min)


B
0
-1
-2
-3
-4
-5
-6
log (N/N0)
0
5
10
15
Contact time (min)

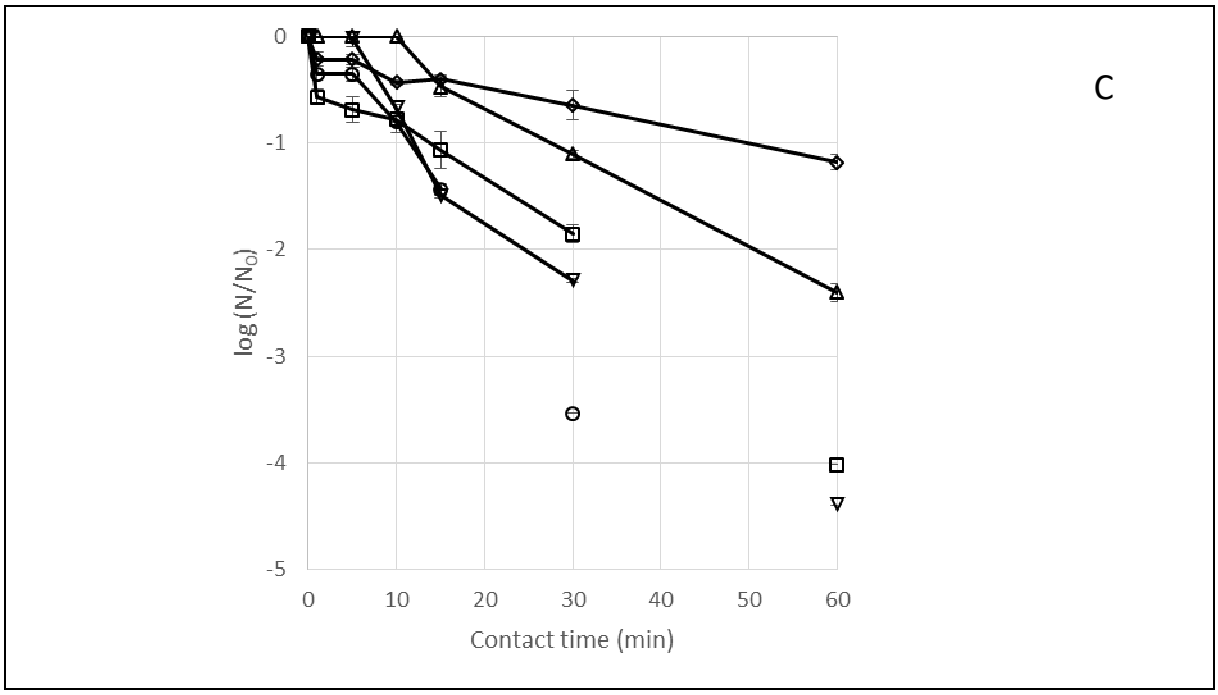


Figure 2

Table 1. List of strains provided by the Université de Bretagne Occidentale Culture Collection (UBOCC).

Species	Reference collection number	Origin
Aspergillus flavus var *columnaris*	UBOCC-A-108066	Butter
Cladosporium cladosporioides	UBOCC-A-111114	Fresh dairy product
Mucor circinelloides	UBOCC-A-112187	Yoghurt
Penicillium commune	UBOCC-A-112059	Blue-veined cheese
Penicillium commune	UBOCC-A-116003	Blue-veined cheese

Table 2. Log$_{10}$ reductions obtained after exposure to various disinfectants using the liquid method.

		Inactivation values (log$_{10}$)									
Mold (Strain)		*Aspergillus flavus* (UBOCC-A-108066)		*Cladosporium cladosporioides* (UBOCC-A-111114)		*Mucor circinelloides* (UBOCC-A-112187)		*Penicillium commune* (UBOCC-A-112059)		*Penicillium commune* (UBOCC-A-116003)	
Physiological state		Labo--ratory	Food plant	Labo--ratory	Food plant	Labo--ratory	Food plant	Labo--ratory	Food plant	Labo--ratory	Food plant
Sodium hypochlorite 0.1% active chlorine	2.5 min	0.0 (± 0.1)	0.2 (± 0.1)	2.8 (± 0.2)	2.7 (± 0.2)	0.1* (± 0.1)	> 4.3*	0.0 (± 0.1)	0.1 (± 0.1)	0.2 (± 0.1)	0.1 (± 0.1)
	5 min	0.2 (± 0.1)	0.1 (± 0.1)	> 3.9	> 4.6	> 3.2	> 4.3	0.0 (± 0.2)	0.4 (± 0.1)	0.3 (± 0.1)	0.2 (± 0.1)
	12.5 min	> 3.7*	0.7* (± 0.2)					1.5* (± 0.1)	3.0* (± 0.2)	3.0 (± 0.2)	3.1 (± 0.1)
Sodium hypochlorite 0.2% active chlorine	1 min					0.2* (± 0.1)	> 4.3*				
	2.5 min	0.5 (± 0.1)	0.4 (± 0.1)	> 4.1	> 4.5	> 3.2	> 4.3	0.1 (± 0.1)	0.2 (± 0.1)	0.2 (± 0.1)	0.2 (± 0.1)
	7.5 min	> 4.3*	0.9* (± 0.1)					1.9* (± 0.1)	4.6* (± 0.9)	3.1 (± 0.1)	3.5 (± 0.2)
Hydrogen peroxide 3%	10 min	3.0* (± 0.1)	0.8* (± 0.1)	0.1 (± 0.1)	0.4 (± 0.1)	0.0* (± 0.1)	0.8* (± 0.1)	> 4.1*	0.0* (± 0.1)	> 4.2*	0.7* (± 0.1)
	15 min	3.6* (± 0.4)	1.4* (± 0.1)	0.1 (± 0.1)	0.4 (± 0.1)	0.1* (± 0.1)	1.1* (± 0.2)	> 4.1*	0.5* (± 0.1)	>4.2*	1.5* (± 0.1)
	60 min	> 4.0	> 3.5	1.4 (± 0.1)	1.2 (± 0.1)	1.3* (± 0.2)	> 4.0*	> 4.1*	2.4* (± 0.1)	> 4.2	> 4.4

"Laboratory": spores produced at 0.99 a_w and harvested using an aqueous solution. "Food plant": spores produced at 0.95 a_w and dry-harvested. Shaded cells were used to compare the physiological states. *: inactivation of "laboratory" spores significantly different from that of "food plant" ones at p=0.05.