Food Microbiology 83 (2019) 59–63

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Antifungal activity of commercial sanitizers against strains of *Penicillium roqueforti*, *Penicillium paneum*, *Hyphopichia burtonii*, and *Aspergillus pseudoglaucus*: Bakery spoilage fungi

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ARTICLE INFO

Keywords:
Sanitizers
Fungal control
Spoilage
Bakeries products*Chemical compounds studied in this article:*
Benzalkonium chloride
biguanide
peracetic acid
quaternary ammonium
sodium hypochlorite

ABSTRACT

Information on the sensitivity of spoilage fungi of bakery products to sanitizing agents is scarce in the literature. Thus, the aim of this study was to evaluate the antifungal activity of different classes of commercial sanitizers, which have permitted use in the food industry, on the main fungi involved in spoiling bakery products. The tests were carried out according to the protocol for testing the antifungal effect of chemical sanitizers of the European Committee for Standardization (CEN), with adaptations. Different strains of six isolated fungal species responsible for spoiling bakery products (*Penicillium roqueforti*, *Penicillium paneum*, *Hyphopichia burtonii*, and *Aspergillus pseudoglaucus*) were tested against five sanitizers at three concentrations: benzalkonium chloride (0.3%, 2.5%, 5%), biguanide (2%, 3.5%, 5%), peracetic acid (0.15%, 1.5%, 3%), quaternary ammonium (0.3%, 2.5%, 5%), and sodium hypochlorite (0.01%, 0.1%, 0.2%). Peracetic acid was the most effective sanitizes considering the genera, species, and concentrations evaluated, generally being capable of reductions between 2 and 4 logs of initial control tested. Biguanide should not be the compound of choice when the main goal of the bakery industry is fungal control.

1. Introduction

Due to high fungal contamination in stored cereals such as wheat and corn (Biro et al., 2009; Chehri et al., 2010; Eglezos, 2010), fungal contamination is extremely important for the bakery industry. Therefore, the consequent deterioration caused by this type of microorganism has become an economically relevant problem for this industry (Pitt and Hocking, 2009).

Fungi are the main spoilage agents of bakery products. They are able to develop in such products due to their intrinsic characteristics, such as intermediate water activity, low acidity, and rich carbohydrate content (Dagnas et al., 2017). The main filamentous fungi involved in contamination and consequent spoilage of bakery products are *Penicillium* and *Aspergillus* (Vytřasová et al., 2002). Likewise, yeast spoilage knew as “chalky mold” is caused by *Hyphopichia* and *Endomyces* (Pitt and Hocking, 2009). Among these genera stand out the species of *Penicillium roqueforti*, *Penicillium paneum*, *Aspergillus pseudoglaucus* (formerly *Eurotium repens*), and *Hyphopichia burtonii* (Dos Santos et al., 2016; Garcia and Copetti, 2018; Morassi et al., 2018).

The fungal spoilage of bakery products begins with the contamination of the cereals in the field by spores that remain viable in the flour. Then, when used as raw material of bakery products, these spores are dispersed in the air and the factory environment, equipment, and surfaces in the form of propagules (Legan, 1993)., This also occurs in a similar way in dairy products and dairy industries (Kure et al., 2004; Vacheyrou et al., 2011). It is believed that all fungal spores present in flour are eliminated during bread baking and similar activities (Garcia et al., 2019). However, propagules dispersed in the air of the processing environment serve as a source of contamination when deposited on the surface of freshly baked products during the cooling stage (Hedrick and Heldman, 1969; Seiler, 1982; Viljoen and Holy, 1997). The hygienic level of the site determines the initial contaminating microbial load, which, together with composition and storage conditions, influences the time for the product to spoil. Visible mycelium formation may occur during storage at the retail location or even at the home of the consumer, which is often even before the shelf-life (Dagnas and Membré, 2013; Horner and Anagnostopoulos, 1973; Lemos et al., 2018).

Reducing the microbial contamination of the production

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https://doi.org/10.1016/j.fm.2019.04.005
Received 2 October 2018; Received in revised form 9 April 2019; Accepted 11 April 2019
Available online 12 April 2019
0740-0020/

environment is one of the most effective ways to minimize losses by spoilage. This can be achieved by adopting hygienic-sanitary measures, such as effective cleaning and sanitization procedures. The most suitable active sanitizing principle for each area and the concentrations employed are the most important factors to be considered in order to achieve the expected objectives (Kuaye, 2017; Rutala, 1996).

Recent studies have shown that knowledge about spoilage fungi species of food products in an industry (target fungi) is relevant when choosing potential sanitizers since differences in fungal sensitivity were observed for the main sanitizers employed by the food industry (Bernardi et al., 2018, 2019).

Thus, the objective of this study was to evaluate the antifungal activity of different classes of commercial sanitizers, which have permitted use in the food industry, on the main species of spoilage fungi of bakery products. Additionally, the existence of differences in the susceptibility of different species and among isolates of the same species in the concentrations used in the tests was also analyzed.

2. Materials and methods

2.1. Microorganisms used and standardization of the initial inoculum

The fungi used in this study are listed in Table 1 and were isolated in their entirety from spoiled bakery products and were identified according to the manual for identification of *Penicillium* species according to Frisvad and Samson (2004) and Pitt (2000); *Aspergillus* and *Hyphopichia* were identified according to Pitt and Hocking (2009). Different strains were tested within the same species in order to verify the existing variations in the susceptibility of the isolates to the tested sanitizers.

To prepare the initial inoculum, tubes containing Malt Extract Agar (MEAc) [glucose, 20 g (Neon, São Paulo, Brazil); peptone, 1 g (Himedia, Mumbai, India); malt extract, 30 g (Bacto ™, MD, USA); solution of trace metals, 1 mL; distilled water, 1 L], were inoculated with each fungal strain, followed by incubation for 7 days at 25 °C. Spores were collected by scraping the mycelium using a sterile aqueous solution of Tween 80 (0.05%). Dilutions were made in 0.1% peptone water [peptone, 0.1 g (Himedia, Mumbai, India); distilled water, 1 L]. The spore concentration was standardized in 10^8 spores/mL with the aid of a Neubauer chamber. Fungal counts were confirmed by inoculation in MEA plates and incubation for 5 days at 25 °C (Bernardi et al., 2018).

2.2. Sanitizers, recommended concentrations, and neutralization

Five different sanitizers from the chemical principles available in the Brazilian market for authorized use in the food industry by the National Health Surveillance Agency (ANVISA) were tested: benzalkonium chloride (0.3%, 2.5%, 5%), biguanide (2%, 3.5%, 5%), peracetic acid (0.15%, 1.5%, 3%), quaternary ammonium (0.3%, 2.5%, 5%), and sodium hypochlorite [0.01% (100 ppm); 0.1% (1000 ppm); 0.2% (2000 ppm)]. The concentration values tested were the minimum and maximum values suggested on the label of the sanitizers, in addition to an intermediate concentration, which was calculated from the mean of the values specified on the label. These principles are also the same generally chosen by the health authorities of the European Union and the United States (CDC, 2008; EPA, 1999; Jeffrey, 1995).

Table 1
Strains used in the commercial sanitizers efficacy test and source of isolation.

Fungi	Strain	Source of isolation
Aspergillus pseudoglaucus	ER 04	Spoiled panettone, Brazil
Aspergillus pseudoglaucus	ER 05	Spoiled panettone, Brazil
Hyphopichia burtonii	HB 100	Spoiled bread, Brazil
Hyphopichia burtonii	HB 17	Spoiled bread, Brazil
Hyphopichia burtonii	HB 08	Spoiled bread, Brazil
Penicillium paneum	LMQA 03	Spoiled bread, Brazil
Penicillium paneum	LMQA 04	Spoiled bread, Brazil
Penicillium paneum	LMQA 05	Spoiled bread, Brazil
Penicillium roqueforti	PR 67	Spoiled bread, Brazil
Penicillium roqueforti	PR 06	Spoiled bread, Brazil
Penicillium roqueforti	PR 11	Spoiled bread, Brazil

To ensure that the action of the sanitizer occurred only during the contact time of the test, neutralization was performed using neutralizing solutions previously tested and indicated in the literature for each sanitizing principle. For the sanitizers, peracetic acid and a sodium hypochlorite solution containing 0.6% of sodium thiosulfate were used. For the other sanitizers tested, nutrient broth with 0.5% Tween 80 and tryptone 1% was employed (Jaenisch et al., 2010).

2.3. In vitro antifungal efficacy of commercial sanitizers

The tests were carried out according to the standards established for the antimicrobial test of chemical sanitizers by the European Committee for Standardization (CEN) with adaptations by Bernardi et al. (2018) (EUROPEAN STANDARD 13697, 2001).

As carriers of the microorganisms, 304 stainless steel discs 2 cm in diameter (TSM inox®, Santa Maria, Brazil) were used. The test was carried out by contaminating five discs with 50 µL of the fungal spore suspension adjusted in 10^7 spores/mL and subsequently added with 0.05% reconstituted skim milk powder (Elegê, São Paulo, Brazil), which simulated the presence of organic matter in the environment. To test the efficacy of the sanitizer (effective sensitivity) on each fungal species, three discs were used. Two discs were used as the positive control (not exposed) of the test.

After the inoculation process, the discs from both tests (effective sensitivity and positive control) were taken to the oven at 35 °C for approximately 40 min for drying and inoculum fixation.

The sensitivity test was performed by adding 100 µL of the sanitizers on each disc containing the dry microbial inoculum at the three different concentrations. To evaluate the positive control, the sanitizer was replaced with 100 µL of sterile water.

Following 15 min of exposure, the discs were immersed in a liquid containing 10 mL of the specific neutralizing solution for each sanitizer. After 5 min of neutralization, serial dilutions were performed in 0.1% peptone water (10^{-1}, 10^{-2}, 10^{-3}) and the inoculation of a 1 mL aliquot in sterile Petri dishes and mixed with 20 mL of Malt Extract Agar (MEA) (malt extract, food grade, 30 g/L, agar 15 g/L) (pour plating).

The plates were incubated at 25 °C for 5 days. Then, the colonies were counted and the results expressed in logarithmic units (log). All tests were performed under aseptic conditions and in duplicate.

2.4. Data analyses

2.4.1. Statistical analyses

Sanitizer efficacy is evaluated by the difference between the number of fungal cells recovered from the positive control (without exposure to the sanitizer) and the microorganisms exposed to the sanitizer.

According to the CEN standard, the sanitizer must reduce, in the case of fungi, 3 log of the initial amount of microorganisms recovered in the positive control in order to be considered an efficient sanitizer (EUROPEAN STANDARD 13697, 2001).

Variance analysis (ANOVA) was performed. Means of fungal recovery after exposure to the commercial sanitizers was analyzed using the Scott-Knott test ($p < 0.05$). Statistical analyses were performed using version 5.6 of SISVAR® Software (Ferreira, 2011).

2.4.2. Antifungal efficacy

To evaluate the antifungal efficacy of the tested sanitizers, the antifungal scale proposed by Bernardi et al. (2018) is adopted. The scale is divided into five comparative parameters:

- maximum efficacy = when reducing the fungal count by at least 4

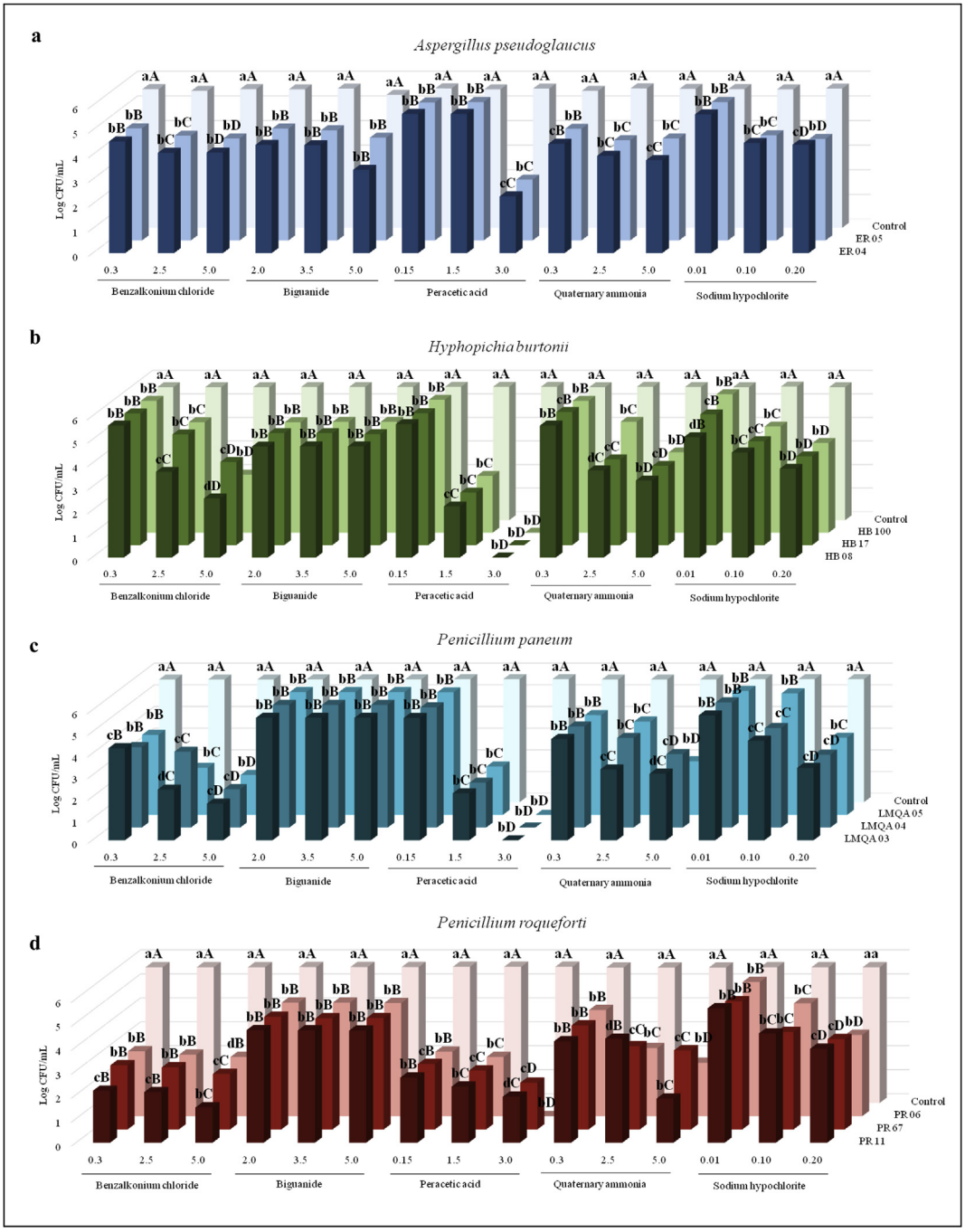

Fig. 1. Efficacy of commercial sanitizers against bakery spoilage fungi: (a) *Aspergillus pseudoglaucus* (ER 04; ER 05); (b) *Hyphopichia burtonii* (HB 08; HB 17; HB 100); (c) *Penicillium paneum* (LMQA 03; LMQA 04; LMQA 05); and (d) *Penicillium roqueforti* (PR 06; PR 11; PR 67) tested with the recommended use concentrations to benzalkonium chloride; biguanide; peracetic acid; quaternary ammonium; sodium hypochlorite. [1]Different lowercase letters in the same column indicate differences between the tested isolates of the same concentration of sanitizers employed according to Scott-Knott test ($p < 0.05$). [2]Different uppercase letters in the same line indicate differences between the concentrations of sanitizers tested for the same isolates according to Scott-Knott test ($p < 0.05$).

log in relation to the positive control;

- good efficacy = fungal count reduced from 3.9 to 3 log;
- reduced efficacy = fungal count reduced from 2.9 to 2 log;
- poor efficacy = fungal count reduced from 1.9 to 1 log;
- inefficacy or no effect = when the microbial population remained unchanged.

3. Results and discussion

Variations were observed in the antifungal activity between the sanitizers tested, the applied concentrations, as well as the susceptibility between the genera, species, and isolates used in the test (Fig. 1 and Supplementary Material Tables 1–5).

Effectively, fungi contamination is one of the most determinative causes of bakery product spoilage, which generally influences the product shelf life. Among the fungal genera, the most commonly associated one with bread spoilage is *Penicillium* (Garcia and Copetti, 2018; Legan, 1993).

Strains of *P. roqueforti* and *P. paneum*, in general, presented differences in sensitivity to the sanitizers used in the test, showing a variation

in the susceptibility pattern for most of the agents at the concentrations used. *P. paneum* and *P. roqueforti* strains showed greater resistance for the lowest concentrations used in the tests with all tested sanitizers. On the other hand, in the peracetic acid, the strains of *P. roqueforti* showed low resistance, as the sanitizer was capable of reducing by practically 3 log (good sanitizing efficacy).

This result is similar to what was reported by Bernardi et al. (2018), in which the sanitizers were not effective at their lowest tested concentrations, although at the largest ones they obtained a reduction between 3 and 5 log of the *P. roqueforti* strain of environmental origin.

A difference was observed between strains of the same species, except for biguanide; between both strains of the same species in relation to the type of sanitizers and between the tested concentrations.

In the *H. burtonii* strains (HB 100, HB 17, and HB 08), a variation was observed in the intermediate concentrations in the tests with benzalkonium chloride (2.5%) and quaternary ammonium (2.5%). In relation to benzalkonium chloride, the strains of *H. burtonii* HB 100 and HB 17 were more resistant than the strain of *H. burtonii* HB 08. Although HB 08 obtained a reduction of 2 log (reduced efficacy), the others did not reach 1 log of reduction (inefficacy) in relation to the positive control for the intermediate concentration tested.

This was the opposite of the quaternary ammonium, where for intermediate concentrations, the lowest resistance was found in the strains of *H. burtonii* HB 17 and *H. burtonii* HB 08, which presented a difference of 1 log reduction in relation to the strain of *H. burtonii* HB 100.

Benzalkonium chloride is an ammonium quaternary compound that belongs to the group of cationic surfactants. This compound attacks microorganisms, causing cell wall lysis. This alters the protein metabolism and causes protein denaturation and enzymatic inhibition (Andrade, 2008; Shaban et al., 2013), which is effective as an active ingredient in antimicrobial products (Shaban et al., 2013), although with a selective germicidal effect (Kuaye, 2017). Benzalkonium chloride is a first-generation ammonium quaternary compound. In relation to the original active principle, it contains a radical of the benzene group (Kuaye, 2017; Tadros, 2005) and its better effectiveness in relation to the original compound may be related to these factors.

Among the different tested strains of *P. roqueforti*, the same is observed in the variation of results among the sanitizers mentioned above, but with greater efficacy in relation to the benzalkonium chloride. Benzalkonium chloride was able to reduce 3 log or more (good efficacy) all strains of *P. roqueforti* (PR 06; PR 11 and PR 67) at all concentrations tested. On the other hand, the *P. roqueforti* strain PR 67 presented higher resistance than the others in relation to quaternary ammonium at the highest concentration this sanitizer was unable to reach the required reduction of 3 log in any of the concentrations used.

The variations between strains also occurred for *P. paneum*, as the quaternary ammonium (0.3%, 2.5%, and 5%) was only effective (log reduction > 3) at the highest concentration (5%) employed and only for the *P. paneum* LMQA 05 strain. In addition, this strain also presented resistance to sodium hypochlorite at the intermediate and higher in both concentrations tested in the study and did not reach the required reduction of 3 log in relation to the positive control.

The variation of results between the strains of *P. roqueforti* and *P. paneum* are relevant as both strains have only recently been classified as *P. roqueforti*. In fact, this species was divided, in the early 90s, into *P. roqueforti*, *P. carneum* and *P. paneum*, which have different rDNA sequences and secondary metabolic profiles (Boysen et al., 2000, 1996).

Because of the increased resistance of these fungi to chemical sanitizers, the characterization and identification of fungal microbiota from the air of the production area and bakery products spoilage are essential for more effective control of the problem.

In relation to the antifungal capacity of the other sanitizers tested, peracetic acid presented the best reductions and consequent sanitizing efficacy. All the strains had low resistance to the intermediate and higher concentrations of peracetic acid employed in the tests. Peracetic acid reduced the initial population of the strains of *P. roqueforti*, *P. paneum,* and *H. burtonii*, from 3 to 5 log (good to maximum efficacy) in relation to the positive control at concentrations of 1.5% and 3% tested.

The peracetic acid used, in addition to its active principle, contains a proportion of acetic acid. Acetic acid can be used in bread and acts as a preservative that reduces the pH of the dough and improves the action of propionic acid, both acting as antifungal compounds (Marin et al., 2003).

In this context, surface treatment with sanitizing agents becomes very relevant (Antolak et al., 2017), and in this case the use of peracetic acid to decontaminate environment air and equipment surfaces are the best way to reduce the loading of contaminants into freshly prepared bakery products and prolong their shelf life.

Sodium hypochlorite, which is regarded as a broad-spectrum and low-cost sanitizer (Kuaye, 2017), is commonly used in food industries in concentrations ranging from 0.02% to 0.08% for equipment and utensils sanitation; and up to 0.12% for facilities cleaning (Menegaro et al., 2016). However, according to our study, these concentrations are not effective against fungal strains isolated from spoiled bakery products. Additional investigation (data not shown) revealed good efficacy of this agent at only 1% concentration, except for *H. burtonii*, which was sensitive at 0.5%. Chlorine solutions are by nature highly corrosive and high concentration solutions can shorten the life of treated equipment. By using different methodologies, such as different dilution tests and exposure times, sodium hypochlorite inhibited the yeast *Saccharomyces cerevisiae* at 0.1% (Winniczuk and Parish, 1997) and *A. niger* at 0.2% concentration (Ozyurt, 2000). In addition, Reynolds et al. (2004) achieved a reduction of more than 5 log cycles of *Penicillium*, *Cladosporium*, *Mucor*, *Rhizopus*, *Alternaria*, and *Aspergillus* in 5 min of exposure to this same agent at a concentration of 2.4%.

As shown in a previous study by Bernardi et al. (2018), biguanide did not demonstrate efficacy in reducing the fungal species used in the tests, which reinforces that this should not be the sanitizer of choice when the objective is fungal control in the bakery industry.

Biguanide hexamethylene and polymer biguanides are the main active components of some products widely used in environment decontamination of the food industry, although their indication is related to hand hygiene (Avecia, 2004).

In addition to the genus *Penicillium* and *H. Burtonii*, xerophilic species of *Aspergillus*, especially those with the *Eurotium* sexual form, are also worth mentioning in bread spoilage. Xerophilic fungi, such as *A. pseudoglaucus* (formerly *E. repens*), are commonly related to spoilage of low water activity products, including bread and bakery products (Antony-Babu and Singleton, 2011; Eicher and Ludwig, 2002; Tranquillini et al., 2017; Vytřasová et al., 2002).

The different strains tested from the *A. pseudoglaucus* species (ER 04 and ER 05) were extremely resistant to the sanitizers. With the exception of the highest concentration of peracetic acid (3%) that reached “good efficacy”, the other agents at the different concentrations only achieved “reduced or poor efficacy” in relation to the positive control for both strains of *A. pseudoglaucus*.

Since most fungi are eliminated during the bread baking stage (Garcia et al., 2019) and some other sweet loaf ingredients, such as burned coconut, undergo the roasting process (190 °C) before being used in gingerbread type bread, these are not considered critical points of fungal control in the bakery industry (Vytřasová et al., 2002). However, if the main fungal load is from heat-resistant fungi (HRMS) due to the production of ascospores (sexual spores), these spores present in the bread or dispersed in the environment air and surfaces by the manipulation may re-germinate and spoil the final product (Rico-Munoz et al., 2019; Tranquillini et al., 2017; Vytřasová et al., 2002).

Ascospore-producing fungi, such as *A. pseudoglaucus*, have resistance to chemical agents commonly used in decontaminating the air and surfaces of the bakery industry. Therefore, it is highly recommended to be familiar with the local microbiota of each industry by performing previous tests with the sanitizers and adjusting the

concentrations to control these agents.

4. Conclusion

Peracetic acid was the most effective sanitizer considering the genera, species, and concentrations evaluated in this study. On the other hand, benzalkonium chloride, and quaternary ammonium presented variable fungal reduction results, both in relation to species, genera, and concentrations. Sodium hypochlorite was not effective in commonly used concentrations and biguanide had very low efficacy and should not be the compound of choice when the main goal of the bakery industry is fungal control. The existence of variations in sensitivity between the species and within the same species was verified, therefore, the isolation and in vitro evaluation of the susceptibility of the problem fungi of each bakery industry to the available sanitizers is recommended.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.04.005.

Funding sources

This study was supported by *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) through doctoral grants to A.O.B. and M.V.G (Finance Code 001), *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) through a research grant to M.V.C. (Process 309691/2015-0 and 428454/2018-6), and scientific initiation fellowships to A.S. and *Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul* (FAPERGS) for scientific initiation fellowships to J.G.L.

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