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Angélica Olivier Bernardi

ESTUDO DA EFICÁCIA ANTIFÚNGICA DE SANITIZANTES PARA CONTROLE DE FUNGOS DETERIORANTES EM INDÚSTRIAS ALIMENTÍCIAS

Santa Maria, RS
2019

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Tese apresentada ao Curso de Pós-Graduação em Ciência e Tecnologia dos Alimentos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutora em Ciência e Tecnologia dos Alimentos.**

Orientadora: Prof.ª Dr.ª Marina Venturini Copetti

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Santa Maria, RS
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DEDICATÓRIA

Aos meus pais José e Patrícia, meus primeiros orientadores

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(Albert Einstein)

RESUMO

ESTUDO DA EFICÁCIA ANTIFÚNGICA DE SANITIZANTES PARA CONTROLE DE FUNGOS DETERIORANTES EM INDÚSTRIAS ALIMENTÍCIAS

AUTORA: Angélica Olivier Bernardi
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Os fungos possuem relevância como agentes de deterioração de alimentos, e o ambiente de produção, incluindo o ar, representam uma importante fonte para contaminação de produtos como pães, queijos, salames e presuntos curados. Assim, a adequada higienização destes locais assume um papel fundamental para prolongar a vida útil destes alimentos. Por outro lado, informações sobre a sensibilidade de fungos deteriorantes de produtos alimentícios a agentes sanitizantes ainda são escassas na literatura. Portanto o objetivo deste estudo foi avaliar a atividade antifúngica de diferentes classes de saneantes comerciais, com uso permitido na indústria de alimentos, frente aos principais fungos envolvidos na deterioração de produtos de panificação, laticínios e produtos cárneos. Os testes foram realizados de acordo com o protocolo para testes de efeitos antimicrobiano de sanitizantes químicos do Comitê Europeu de Normalização (CEN) e da Norma Francesa NF-T-72281. Espécies dos gêneros *Aspergillus (A. brasiliensis, A. flavus, A. westerdijkiae, A. pseudoglaucus, A. chevalieri)*, *Penicillium (P. roqueforti, P. commune, P. polonicum, P. paneum)*, e espécies de *Hyphophicia burtonii, Candida albicans, Cladosporium cladosporioides, Mucor hiemalis* e *Lichtheima corymbifera* foram testadas frente a cloreto de benzalcônio (0,3%, 2,5%, 5%), biguanida (0,3%, 2,5%, 5%), ácido peracético (0,15%, 1,5%, 3%), quaternário de amônia (0,3%, 2,5%, 5%) hipoclorito de sódio (0,01%, 0,1%, 0,2%, 0,5%, 1%) e um agente gerador de fumaça à base de ortofenilfenol 15% (1g/m^3). O ácido peracético foi o sanitizante mais eficaz considerando todas as espécies fúngicas testadas, seguido pelo cloreto de benzalcônio e amônia quaternária, porém a eficácia destes agentes somente ocorreu nas doses mais altas recomendadas pelo fabricante. A biguanida, o hipoclorito de sódio e o agente gerador de fumaça foram os sanitizantes menos eficazes dentre todos os avaliados no estudo. De maneira geral, houve variação entre as espécies de fungos testadas e também entre cepas de uma mesma espécie, principalmente se tiverem sido isoladas de substratos distintos. Fungos deteriorantes de produtos cárneos foram os que apresentaram maior resistência aos sanitizantes avaliados. Assim, o conhecimento da eficácia antifúngica dos principais agentes sanitizantes permitidos na indústria alimentícia frente às principais espécies de fungos envolvidos na deterioração de alimentos pode auxiliar na escolha do melhor agente para a higiene industrial visando o controle fúngico em cada segmento específico e, portanto, colaborar na redução de perdas econômicas ocasionadas por fungos.

Palavras-chave: Deterioração de alimentos. Qualidade do ar ambiente. Higienização. Higiene industrial. Sanitizantes. Controle microbiológico.

ABSTRACT

STUDY OF THE ANTIFUNGAL EFFICACY OF SANITIZERS TO CONTROL SPOILAGE FUNGI IN THE FOOD INDUSTRY

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ADVISOR: Prof.ª Dr.ª Marina Venturini Copetti

Fungi have relevance as agents of food spoilage, and the production environment, including air, is an important source for contamination of products such as bread, cheeses, salami and, cured hams. Thus, the adequate hygiene of these places plays a fundamental role in extending the useful life of these foods. On the other hand, information on the sensitivity of deteriorating fungi from food products to sanitizing agents is still scarce in the literature. Therefore, the aim of this study was to evaluate the antifungal activity of different classes of commercial sanitizers, with the permitted use in the food industry, against the main fungi involved in the spoilage of bakery products, dairy products, and meat products. The tests were carried out according to the protocol for testing the antimicrobial effects of chemical sanitizers of the European Committee for Standardization (CEN) and French Standard NF-T-72281. Species of the genus *Aspergillus* (*A. brasiliensis*, *A. flavus*, *A. westerdijkiae*, *A. pseudoglaucus*, *A. chevalieri*), *Penicillium* (*P. roqueforti*, *P. commune, P. polonicum*, *P. paneum*) and species of *Hyphophicia burtonii, Candida albicans*, *Cladosporium cladosporioides*, *Mucor hiemalis* and, *Lichtheima corymbifera* were tested against benzalkonium chloride (0.3%, 2.5%, 5%), biguanide (0.3%, 2.5%, 5%), peracetic acid (0.15%, 1.5%, 3%), quaternary ammonia (0.3%, 2.5%, 5%) sodium hypochlorite (0.01%, 0.1%, 0, 2%, 0.5%, 1%) and a smoke generating agent based on orthophenylphenol 15% (1g/m^3). Peracetic acid was the most effective sanitizer considering all fungal species tested, followed by benzalkonium chloride and quaternary ammonia, but the efficacy of these agents only occurred at the highest doses recommended by the manufacturer. Biguanide, sodium hypochlorite, and the fuming agent were the least effective sanitizers among all those evaluated in the study. In general, there was variation between the species of fungi tested and also between strains of the same species, especially if they were isolated from different substrates. Spoilage fungi of meat products were the ones that presented greater resistance to the evaluated sanitizers. Thus, the knowledge of the antifungal efficacy of the main sanitizing agents allowed in the food industry against the main species of fungi involved in the spoilage of food can help in the choice of the best agent for industrial hygiene, aiming the fungal control in each specific segment and, therefore, collaborate in the reduction of economic losses caused by fungi.

Keywords: Food spoilage. Environment air quality. Sanitation. Industrial hygiene. Sanitizers. Microbiological control.

LISTA DE ILUSTRAÇÕES

5.5 ARTIGO 5

LISTA DE TABELAS

LISTA DE QUADROS

REVISÃO BIBLIOGRÁFICA

SUMÁRIO

APRESENTAÇÃO

A presente tese de doutorado segue as normas estabelecidas na Estrutura e Apresentação de Monografias, Dissertações e Teses – MDT da UFSM (UFSM, 2015). Os resultados obtidos ao longo dos anos de doutoramento estão apresentados na forma de cinco artigos científicos, sendo quatro com resultados da pesquisa e um de revisão bibliográfica.

O primeiro artigo integrado apresentará um artigo publicado na revista LWT-Food Science and Technology no ano de 2018 intitulado "Efficacy of commercial sanitizers against fungi of concern in the food industry" que apresenta informações de maneira mais geral de resultados de eficácia de sanitizantes comerciais comumente utilizados e de uso autorizado pela legislação brasileira e estrangeira frente a fungos relacionados à deterioração fúngica de alimentos, além de fungos indicadores de qualidade ambiental em indústrias alimentícias.

No mesmo caminho, o segundo artigo traz um estudo realizado com os mesmos fungos, porém com a inovação de serem confrontados com um sanitizante gerador de fumaça, e que foi publicado pelo International Journal of Food Microbiology em 2019, intitulado "Sensitivity of food spoilage fungi to a smoke generator sanitizer". A partir destes resultados foi definida a sequência da pesquisa avaliando diferentes segmentos de indústrias de alimentos que geralmente apresentam problemas com deterioração por fungos filamentosos, utilizando-se cepas fúngicas isoladas de produtos deteriorados.

Assim, o terceiro artigo apresenta um estudo sobre a eficácia de sanitizantes frente a fungos deteriorantes de produtos panificados, o qual foi publicado em 2019 pela revista Food Microbiology nomeado "Antifungal activity of commercial sanitizers against strains of *Penicillium roqueforti*, *Penicillium paneum*, *Hyphopichia burtonii*, and *Aspergillus pseudoglaucus*: Bakery spoilage fungi".

Na mesma linha o quarto artigo trará um manuscrito recentemento submetido a revista Food Research International que trata da eficácia de sanitizantes líquidos e um gerador de fumaça frente a fungos deteriorantes de queijos e produtos cárneos curados.

Finalizando os capítulos dessa tese, no quinto artigo integrado a tese será encontrado um manuscrito de revisão bibliográfica intitulado "Food industry spoilage fungi control through facility sanitization", submetido para uma edição especial de

Food Micology da revista Current Opinion in Food Science e que discute os resultados obtidos ao longo dos quatro capítulos anteriores.

Ao final desta tese, encontram-se os itens discussão geral e conclusões, apresentando uma compilação de interpretações e comentários a respeito dos resultados apresentados nos artigos científicos, ilustrado através da construção de um *heatmap*.

1 INTRODUÇÃO

A deterioração de alimentos é um grande problema para a indústria alimentícia causado pela contaminação do produto, prévia ou posteriormente ao processamento, por esporos fúngicos e que em condições intrínsecas favoráveis, como atividade de água (a_w) e pH, germinarão formando um micélio visível, consequentemente deteriorando o produto antes mesmo deste ter extrapolado o seu prazo de validade. Além disso, em alguns casos pode causar danos a saúde dos consumidores através da produção de micotoxinas pelos fungos que se desenvolvem no produto (FILTENBORG et al., 1996, PITT; HOCKING, 1999; SAMSON et al., 2004; DAGNAS; MEMBRÉ, 2013; GOUGOULI et al., 2011; GARNIER et al., 2017).

A contaminação fúngica e consequente deterioração de alimentos mais suscetíveis como pães, queijos, produtos cárneos curados, vegetais e outros, ocorre principalmente pelo fato desses produtos possuírem os parâmetros intrínsecos já citados, impeditivos ao desenvolvimento da maioria das bactérias. Dentro desse contexto se destacam geralmente os fungos do gênero *Aspergillus*, *Penicillium*, *Candida*, *Cladosporium* e *Fusarium* (DEÁK, 2008; PITT; HOCKING, 2009).

O ar tem sido descrito como a principal fonte de contaminação principalmente em queijos, produtos cárneos e produtos de panificação (BATTILANI et al., 2007; SØRENSEN et al., 2008; JAHN et al., 2017; WIGMANN, et al., 2018; GARCIA et al., 2019a; PARUSSOLO et al., 2019a). Sendo que este problema ocorre principalmente nos períodos pós-processamento, sobretudo durante o período de resfriamento, no caso dos queijos, estocagem, no caso dos vegetais, período de maturação, no caso dos produtos cárneos e resfriamento no caso dos pães (SNOWDON, 1990; HOCKING; FAEDO, 1992; MUHAMMAD et al., 2004; GARCIA et al., 2019a).

A preocupação com a qualidade micológica do ar e do ambiente fabril é, portanto, um fator que deve ser considerado na busca pelo aumento da vida útil de produtos alimentícios. Portanto, para o controle da contaminação e prevenção da contaminação precoce de alimentos, faz-se necessário a aplicação de um efetivo método de limpeza, seguido de um processo de sanitização ambiental e de superfícies, visando a redução da carga microbiana incial (KUAYE, 2017). Contudo os dados relacionados à avaliação da eficácia de sanitizantes comerciais, utilizando metodologias oficiais, frente a fungos de interesse em alimentos, indicação de

concentrações de uso destes produtos conforme a espécie fúngica envolvida no problema, bem como variações de resistência entre espécies semelhantes são escassos na literatura. Desta maneira, a realização deste estudo é um importante passo para preencher algumas lacunas relevantes para nortear de maneira mais eficaz o processo de higienização dentro das indústrias de alimentos, e desta maneira controlar perdas por deterioração fúngica.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a eficácia antifúngica de sanitizantes comerciais de uso autorizado em indústrias de alimentos frente a fungos filamentosos relacionados à deterioração de alimentos.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar *in vitro* a eficácia individual de cinco sanitizantes líquidos mais empregados em indústrias de alimentos (ácido peracético; biguanida; cloreto de benzalcônio; hipoclorito de sódio e quaternário de amônia) em concentrações de uso recomendada pelo fabricante frente a fungos potencialmente deteriorantes de alimentos.
- Testar *in vitro* a eficácia de um agente saneante dispersado por via aérea a base de ortofenifenol, conforme indicações e recomendações de uso do fabricante frente a espécies com potencial deteriorante em alimentos.
- Verificar a possível ocorrência de variação na sensibilidade de cepas de uma mesma espécie fúngica à diferentes concentrações de sanitizantes.
- Elaborar uma escala de eficácia antifúngica de sanitizantes comerciais conforme as espécies fúngicas avaliadas para facilitar a classificação de ação dos compostos disponíveis.

3 REVISÃO BIBLIOGRÁFICA

3.1 DETERIORAÇÃO DE ALIMENTOS: FONTES E FATORES

O desenvolvimento de fungos indesejáveis pode ocasionar impactos econômicos negativos para os produtores, aumentando as perdas e os custos de produção, além de problemas à saúde do consumidor decorrentes da exposição às micotoxinas, produzidas com o crescimento de fungos toxigênicos (MINTZLAFF et al., 1972; MONACI et al., 2005; TOSCANI et al., 2007; IACUMIN et al., 2009).

Desde as décadas passadas a redução do uso de conservantes na indústria de alimentos vem ocorrendo, principalmente devido à exigência de consumidores que buscam uma alimentação voltada ao consumo orgânico e sustentável. Porém, isso pode culminar no aumento da ocorrência de casos de deterioração de produtos. As causas mais frequentes de deterioração em alimentos industrializados, como pães, queijos e produtos cárneos maturados são os fungos filamentosos e leveduras (BUNDGAARD-NIELSEN; NIELSEN, 1995).

Alguns estudos vêm sendo realizados no intuito de determinar fatores que durante a produção de alimentos industrializados levam a contaminação por bolores indesejados (KURE et al., 2001). O ar tem sido descrito como a principal fonte de contaminação em queijos, produtos de panificação e em produtos cárneos (BATTILANI et al., 2007; SORENSEN et al., 2008; DOS SANTOS et al., 2016; JAHN et al., 2017; WIGMANN et al., 2018; MORASSI et al., 2018; GARCIA et al., 2019b; PARUSSOLO et al., 2019a). A qualidade da matéria-prima e alguns outros fatores físicos como atividade de água e temperatura também são reportados como sendo fatores importantes para o desenvolvimento fúngico em produtos alimentícios (PITT; HOCKING, 1999; MIZAKOVA et al., 2002; BIRO et al., 2009; CHEHRI et al., 2010; EGLEZOS, 2010).

O quadro abaixo ilustra as principais indústrias afetadas com a deterioração fúngica de produtos, bem como as características dos produtos, principais espécies fúngicas envolvidas, fontes de contaminação e do problema.

Quadro 1: Características intrínsecas e fontes de contaminação de fungos deteriorantes de acordo com o tipo de indústria.

Indústria	Características intrínsecas dos produtos	Fungos deteriorantes	Fontes de contaminação	Fontes do problema
Panificação	Ricos em carboidratos, conteúdo de umidade em torno de 40%, atividade de água ~média de 0,94 e 0.98 e acidez ntermediária (pH = 5,5–6,0).	*P. roqueforti*, *P. paneum*, *P. polonicum,* *P. citrinum*, *P. paxilli*, *P. glabrum*, *A. pseudoglaucus,* *A. flavus,* *A. sydowii,* *A. niger,* *C. sitophila* , *H.burtonii*, *E. fibuliger,* *W. sebi.*	- Matérias primas (cereais contamnados no campo, e esporos permanecem viáveis na farinha); - Ar do ambiente de processamento.	- Fungos presentes das farinhas dispersos no ar na forma de aerossóis; - Aerossol serve de fonte de contaminação quando depositado na superfície de pães recém assados durante a etapa de resfriamento (recontaminação); - Habilidade natural dos fungos de sobrepor a barreira de conservantes.
Laticínios	Ricos em lipídeos, pH típico de 4,3-4,9, umidade em torno de 30% para queijos duros; 50% queijos macios; 50% margarinas; 70 a 80% iogurtes, atividade de água variável (0,93 a 0,98), potencial de oxi-redução.	*P. commune*, *P. roqueforti,* *P. polonicum*, *P. glabrum,* *P. chrysogenum,* *P. solitum,* *P. verrucosum,* *Aspergillus* sp., *C. famata*, *C. parapsilosis,* *M. hiemalis,* *G. candidum.*	- Ambiente de produção: ar, superfícies de trabalho, equipamentos, pessoal, matérias primas e ingredientes.	- Baixo nível higiênico do ambiente. - Esporos dispersos no ar se depositam sobre os produtos recém acabados. - O ar das salas de maturação.
Carnes	Ricos em proteínas, carboidratos e gorduras. Média final de pH (4,40-4,80), umidade média (30%-36%), atividade de água média (0,77-0,83).	*A. westerdijkiae,* *A. ochraceus,* *A. candidus,* *P. polonicum,* *P. nordicum,* *P. verrucosum,* *P. nalgiovense,* *P. glabrum,* *C. cladosporioides,* *M. hiemalis,* *D. hansenii,*	- Ambiente de produção: ar, superfícies de trabalho, equipamentos, pessoal, matérias primas e ingredientes.	- Baixo nível higiênico do ambiente. - Dispersão de esporos no ar. - Condições ambientais das salas de maturação. - Tolerância dos fungos ao baixo pH e e altas quantidades de sal.

Fonte: o autor

3.1.1 Deterioração fúngica de pães

Os fungos estão fortemente relacionados à deterioração de pães devido a sua composição [conteúdo de umidade em torno de 40%, atividade de água de 0,94 a 0,98, acidez intermediária (pH = 5,5–6,0)] e a temperatura a qual estes produtos

são expostos para a comercialização (20–35 °C); esses fatores resultam em uma vida de prateleira de 3 a 7 dias para pães que não contêm conservantes (LEGAN, 1993; SARANRAJ; GEETHA, 2012) estendendo-se por até 14 dias para pães que contêm propionado de cálcio, principal conservante utilizados em panificados (GARCIA et al., 2019b).

A deterioração de pães por fungos ocorre a partir do aparecimento do micélio visível sobre a superfície do produto, ocasionando rejeição pelo consumidor (BAERT et al., 2007; DAGNAS; MEMBRÉ, 2013; LEMOS et al., 2018). A formação visível de micélio pode ocorrer durante o armazenamento no local de comercialização ou até mesmo na casa do consumidor, muitas vezes antes mesmo do prazo de validade haver expirado (DAGNAS; MEMBRÉ, 2013; HORNER; ANAGNOSTOPOULOS, 1973). Além da mudança no aspecto visual dos produtos panificados deteriorados, os fungos são responsáveis por alterações em outras características sensoriais como sabor e aroma dos produtos em virtude da produção de exoenzimas, como lipases, proteases e carboidrases (FILTENBORG et al., 1996; NIELSEN; RIOS, 2000).

Devido à alta incidência de contaminação fúngica em cereais estocados, como trigo e milho (BIRO et al., 2009; CHEHRI et al., 2010; EGLEZOS, 2010), a contaminação por fungos se torna extremamente importante para a indústria de panificação e a consequente deterioração precoce causada por esse tipo de micro-organismo tornou-se um problema economicamente relevante para essa indústria (PITT; HOCKING, 2009).

O problema da deterioração fúngica dos produtos de panificação começa com a contaminação dos cereais no campo por esporos que permanecem viáveis na farinha. Então quando esta é usada como matéria-prima de produtos de panificação dentro da indústria, esses esporos são dispersos no ar, no ambiente da fábrica, equipamentos e superfícies na forma de propágulos (LEGAN, 1993).

Acredita-se que todos os esporos fúngicos presentes na farinha são eliminados durante o processo de fabricação (GARCIA et al., 2019b). No entanto, os propágulos dispersos no ar do ambiente de processamento servem como fonte de contaminação quando depositados na superfície de produtos acabados durante o estágio de resfriamento (HEDRICK; HELDMAN, 1969; SEILER, 1982; VILJOEN; HOLY, 1997).

O nível de esporos de fungos no ar de indústrias de panificação costuma variar entre 100 a 2500 esporos/m^3. Este influenciará na carga microbiana contaminante inicial, que se depositará no produto após o resfriamento que sucede ao assamento. Juntamente com as condições de composição e armazenamento, influenciará o tempo de deterioração do produto e poderá predispor à deterioração precoce (CAUVAIN; YOUNG, 2007).

Os principais gêneros de fungos filamentosos envolvidos na contaminação e consequente deterioração dos produtos de panificação são *Penicillium* e *Aspergillus* (VYTRASOVA et al., 2002). Da mesma forma, existe a deterioração causada por leveduras, problema conhecido como "mofo branco", que é causado por fungos leveduriformes do gênero *Hyphopichia* e *Endomyces* (PITT; HOCKING, 2009). Destacam-se as espécies *Penicillium roqueforti*, *Penicillium paneum*, *Aspergillus pseudoglaucus* (anteriormente denominada *Eurotium repens*) e *Hyphopichia burtonii* (DOS SANTOS et al., 2016; GARCIA et al., 2019b; MORASSI et al., 2018).

Além disso, alguns fungos são capazes de resistir a tratamentos químicos e também ao uso de certos conservantes. *Penicillium roqueforti* é usado como micro-organismo indicador em ensaios antifúngicos devido sua alta resistência aos conservantes químicos (CODA et al., 2008), e amplamente relacionado à deterioração de produtos de panificação. Por exemplo, alguns micro-organismos do gênero *Penicillium*, podem crescer na presença de sorbato de potássio (DAVIDSON, 2001) e outros fungos possuem a capacidade de degradar o sorbato (NIELSEN; DE BOER, 2000).

3.1.2 Deterioração fúngica de queijos

Queijos moles e duros são os mais afetados por fungos filamentosos e leveduras (HOCKING; FAEDO, 1992; KURE et al., 2004; LEDENBACH; MARSHALL, 2010). Os gêneros fúngicos envolvidos na deterioração desses produtos se originam principalmente do ambiente de produção, que inclui ar, superfícies de trabalho, equipamentos, pessoal, matérias-primas e ingredientes (KURE et al., 2004; VACHEYROU et al., 2011; JAHN et al., 2017). Como resultado, a presença de fungos na indústria de laticínios é responsável por até 5% da perda de produção (RESA et al., 2014).

Os fungos e leveduras são as maiores fontes de deterioração dos produtos lácteos. No entanto, poucos estudos investigaram a diversidade de fungos nesses produtos (GARNIER et al., 2017). Entre as 41 espécies de fungos isoladas de produtos lácetos deteriorados analisados por Garnier et al. (2017), *P. commune* representou cerca de 10% do total. Em outro estudo, Kure et al. (2004) encontraram *P. roqueforti* ss. *roqueforti* em amostras de ar e equipamentos de indústrias de laticínios. Além disso, *P. commune* também foi encontrado em amostras de embalagens de filmes plásticos. Jahn et al. (2017) também mostraram a presença de *P. commune* no ar de produção de uma indústria de laticínios com problemas de deterioração de queijo tipo tropical.

A faixa típica de pH (4,3 - 4,9) de queijo e teor de umidade favorece o desenvolvimento de fungos filamentosos (JACOBSEN; NARVHUS, 1996). As indústrias buscam controlar esses micro-organismos adicionando compostos antimicrobianos e conservantes durante a fabricação do produto (COSTA et al., 2017). Os conservantes mais comuns utilizados no controle de fungos e leveduras em produtos lácteos são o ácido sórbico e seus sais (LUCERA et al., 2012). O sorbato de potássio é amplamente utilizado devido à sua capacidade de prevenir fungos indesejáveis sem alterar o sabor, o odor e a cor do produto final (KARABULUT et al., 2001; LUCERA et al., 2014).

3.1.3 Deterioração fúngica de produtos cárneos

As partículas fúngicas são consideradas as principais causas de deterioração em produtos de carnes desidratadas e curadas (ROJAS et al., 1991,. NÚÑEZ et al., 1996; PEINTNER et al., 2000; COMI et al., 2004; TABUC et al., 2004; WANG et al., 2006; BATTILANI et al., 2007; PAPAGIANNI et al., 2007; SØRENSEN et al., 2008; ASEFA et al., 2009; PERRONE et al., 2015; PARUSSOLO et al., 2019a).

Sørensen et al. (2008) analisaram as áreas de processamento de linguiças fermentadas onde avaliaram a qualidade do ar, equipamentos e matérias-primas. Foram identificados pelo menos 17 gêneros diferentes como *Penicillium*, *Aspergillus* e *Eurotium*, e das espécies de *Penicillium* isoladas várias eram potenciais produtoras de micotoxinas, como o *P. brevicompactum.*

Em trabalho realizado por Asefa et al., (2010) que investigou o padrão de crescimento fúngico em produtos de carnes curadas verificou que das amostras de ar ambiente analisadas os fungos estavam presente em 80% delas onde mais de 39 espécies diferentes de fungos foram encontradas e destas 77% correspondiam ao gênero *Penicillium* predominando o *P. nalgiovense* que em testes preliminares foi capaz de produzir penicilina. A qualidade do ar da área de salga e as salas de defumação foram apontadas como principais responsáveis pela contaminação fúngica.

O desenvolvimento de fungos indesejáveis pode ocasionar impactos econômicos negativos para os produtores, aumentando as perdas e os custos de produção, além do risco à saúde do consumidor com o crescimento de fungos toxigênicos (MINTZLAFF et al., 1972; MONACI et al., 2005; TOSCANI et al., 2007; IACUMIN et al., 2009). Estes impactos econômicos negativos podem ser minimizados através da redução do crescimento fúngico e atenção aos fatores que levam ao seu desenvolvimento em produtos cárneos curados (ASEFA et al., 2010).

Os gêneros *Penicillium* e *Aspergillus* são comuns isolados de produtos cárneos curados (NÚÑEZ et al., 1996; LARSEN et al., 2001) e dentro desses gêneros algumas espécies, como *Aspergillus westerdijkiae* são capazes de produzir micotoxinas e nesse caso em particular, ocratoxina A (TOSCANI et al., 2007). Este fungo pode ser facilmente confundido com *A. ochraceus* e outros da seção Circumdati porque as características da colônia são muito similares, e muitos fungos que foram previamente indentificados como sendo *A. ochraceus* são atualmente *A. westerdijkiae* (FRISVAD et al., 2004). A presença de *A. ochraceus*/*A. westerdijkiae* tem sido reportada na superfície de produtos cárneos curados (CASTELLARI et al., 2010; IACUMIN et al., 2011; VILA et al., 2016). Scaramuzza et al. (2015) também relataram uma baixa quantidade desta espécie no ar de 1 em 3 instalações de processamento de carnes na Itália, e, recentemente, foi relatado como produtor de micotoxinas em presunto curado (VIPOTNIK et al., 2017).

Uma vez que a presença de *A. westerdijkiae* apresenta um risco em relação a produção de ocratoxina A, considerada a micotoxina mais relevante em produtos cárneos curados, uma limpeza cuidadosa no ambiente de produção e maturação é crucial (PARUSSOLO et al., 2019b).

Nesse sentido, podemos perceber que o controle do ar ambiente de câmaras de maturação de produtos cárneos curados é uma das melhores formas de conseguir o controle de fungos indesejados.

3.2 MÉTODOS DE CONTROLE DA CONTAMINAÇÃO FÚNGICA

A deterioração por fungos em pães, por exemplo, é retardada pela adição de conservantes químicos, como ácidos propiônico, sórbico, acético e seus sais, geralmente reconhecidos como seguros (GRAS). Estes são usados para suprimir o crescimento de micro-organismos e para prolongar a vida útil dos produtos de padaria (GOULD, 1996; LEGAN, 1993).

Nos produtos lácteos como nos queijos também é comum o uso de ácido sórbico e seus sais (LUCERA et al., 2012). O sorbato de potássio é amplamente utilizado devido à sua capacidade de prevenir fungos indesejáveis sem alterar o sabor, o odor e a cor do produto final (KARABULUT et al., 2001; LUCERA et al., 2014), isso também ocorre no caso dos produtos cárneos maturados, como salames e presuntos.

No entanto, as demandas dos consumidores por produtos livres de conservantes nos últimos anos aumentaram, uma vez que requisitos alimentares mais saudáveis e seguros estipularam ingredientes mais naturais em alimentos processados (RESA, et al., 2014). Como resultado, a higiene do ar ambiente e superfícies de trabalho ganharam destaque.

Kuaye (2017) enfatizou que a redução da contaminação no ambiente de produção é uma das formas mais eficazes de reduzir a contaminação inicial (carga microbiana) dos produtos, garantindo vida útil e reduzindo as perdas. Ao adotar medidas higiênico-sanitárias, como empregar métodos eficazes de limpeza e processos de sanitização, é possível reduzir a contaminação de superfícies e ambientes a um nível seguro, o que afetará a carga microbiana dos alimentos e influenciará seu prazo de validade.

3.2.1 Uso de sanitizantes para controle de fungos indesejados

Para assegurar a elaboração de produtos de qualidade, livres de contaminações por agentes químicos, físicos e microbiológicos, a indústria de

alimentos deve estabelecer limites de segurança que possam ser monitorados, mensurados e registrados; de maneira a assegurar que o procedimento seja efetivo e o que foi objetivo estabelecido seja atingido (ANDRADE et al., 2008).

É fundamental que tanto o agente quanto o processo de higienização empregados possibilitem a produção de alimentos com vida-útil prolongada e inócuos à saúde dos consumidores (HAYES, 1993; MORELLI, 2008).

Para a eficácia do processo, é fundamental a seleção de sanitizantes contendo princípios ativos eficazes contra os micro-organismos alvo e que as concentrações utilizadas sejam suficientes para a inativação microbiana sem desperdícios desnecessários, respeitando-se as recomendações de qualidade microbiológica estabelecidas com critério técnico para superfícies higienizadas, ambientes de processamento, manipuladores e equipamentos (ANDRADE et al., 2008).

Um aspecto essencial para que um processo de higienização seja eficaz é a escolha correta do desinfetante a ser utilizado. Para tanto, deve-se levar em conta alguns fatores como espectro de ação, atividade antimicrobiana e também que o desinfetante atenda os requisitos de segurança e legalidade conforme os órgãos fiscalizadores pertinentes. A escolha deve ser feita considerando a superfície e o local a ser desinfetado, o conteúdo de matéria orgânica residual, a temperatura, a quantidade de água, o tempo de contato, o espectro de ação e poder residual do produto, entre outros (KUANA, 2009). Todavia, é comum os desinfetantes serem empregados em concentrações inadequadas, ou haver a mistura de mais de um produto, levando a formulações que podem afetar a atividade antimicrobiana do produto.

3.2.2 Princípios sanitizantes de uso autorizado

Dentre os princípios autorizados (BRASIL, 2007), existem os compostos clorados, compostos iodados, compostos de amônia quaternária, ácido peracético e peróxido de hidrogênio, que são mais viáveis economicamente e mais comumente utilizados pelas indústrias. Há outros compostos que podem ser usados em formulações de sanitizantes, como aldeídos, fenóis, biguaninas e alcoóis (ASSELT; GIFFEL, 1998), como podemos observar no quadro abaixo (Quadro 2). Esses

compostos são os mesmos autorizados para uso pelos Estados Unidos e União Européia (JEFFREY, 1995; WHITE, 1999; CDC, 2008).

Quadro 2: Princípios sanitizantes autorizados no Brasil.

GRUPOS	PRINCÍPIOS ATIVOS
Aldeídos	Formaleído, glicoxal, glutaraldeído e paraformaldeído.
Fenólicos	Tercamilfenol; 2-benzil 4-clorofenol; 4-tercbutilfenol; cresóis 2-fenilfenol; 2-hidroxidifenileter e 2-hidroxi-2, 4, 4-triclorodifenileter.
Quaternários de amônio	Cloreto de alquil dimetil benzil amônio; cloreto de alquil dimetil etilbenzil amônio; cloreto de alquil dimetil etiltoluil amônio; cloreto de lauril piridínio; cloreto e brometo de cetil trimetil amônio; cloreto de alquil trimetil amônio; N, N dialquil N,N dimetil amônio; dicloreto de polioxietileno (dimetilimino) etileno (dimetilimino) e dicloreto de polioxietileno (dimetilimino) metileno (dimetilimino) etileno. Obs.: os radicais alquila estão compreendidos entre C8 e C18, sendo os mais efetivos os produtos resultantes da cominação C12 e C14.
Compostos inorgânicos liberadores de cloro ativo	Hipoclorito de sódio, de lítio e de cálcio.
Compostos orgânicos liberadores de cloro ativo	Ácido diclorocianúrico e os sais de sódio e potássio; ácido triclorocianúrico; N, N-dicloroazodicarbonamida; N-cloro benzenossulfonamida sódica; N-cloro 4-metil benzenossulfonamida sódica; cloro suocinimida e 1,3-dicloro 5,5-dimetil hidantoína.
Iodo e derivados	Iodo, iodo-povidona (PVP-I) e iodóforos.
Biguanidas	Clorohexidina, cloridrato de polihexametileno biguanida.
Peróxido inorgânico	Peróxido de hidrogênio.
Peróxido orgânico	Ácido peracético.

Fonte: ANDRADE (2008).

Nas indústrias alimentícias, os compostos clorados podem ser utilizados para a sanificação de superfícies e utensílios, com a finalidade de reduzir o número de micro-organismos presentes em carcaças bovinas, suínas e de aves, para reduzir a quantidade de micro-organismos que pode estar presentes em frutas e vegetais

minimamente processadas e para controle microbiológico de água (WEI et al., 1995).

O hipoclorito de sódio surgiu como o primeiro saneante para uso em descontaminação ambiental de indústrias de alimentos e estudos de sua eficiência na redução microbiana datam da década de 1920. Contudo desvantagens do uso desse desinfetante logo foram apontadas, entre elas, problemas de estabilidade e eficácia, que duram apenas alguns minutos, além de possuir um conhecido potencial corrosivo em concentrações elevadas (MENEGARO et al., 2016).

O ácido peracético é obtido através da reação do ácido acético ou anidrido acético com o peróxido de hidrogênio. O ácido peracético é um excelente saneante pela grande capacidade de oxidação dos componentes celulares dos micro-organismos, tendo uma rápida ação mesmo em baixas concentrações sobre um amplo espectro de micro-organismos. É esporicida em diferentes temperaturas e continua efetivo mesmo na presença de matéria orgânica, sendo considerado um biocida efetivo sem efeito residual tóxico. Sua ação bactericida ou fungicida é influenciada pela concentração, temperatura e tipo de micro-organismos (NASCIMENTO, 2002).

Os compostos quaternários de amônia são tensoativos catiônicos que apresentam atividade germicida mais relevante do que sua capacidade de atuar como detergente. O cátion é um radical orgânico e o ânion, um halogênio e a mudança desses radicais dá origem a um grande número de produtos com atividade antimicrobiana. Os compostos quaternários de amônia atuam com maior eficiência sobre bactérias Gram positivas, fungos filamentosos e leveduras (ANDRADE; MACEDO, 1996).

O cloreto de benzalcônio é um composto catiônico de quaternário de amônio que possui ação umectante e detergente, com propriedades emulsificadora e germicida. Sua atividade bactericida datada de 1935 propiciou o uso comercial deste saneante (SOUZA-MACHADO et al., 2008).

Os desinfetantes fenólicos, derivados do fenol, como o ortofenilfenol ($C_{12}H_{10}O$) são germicidas de amplo espectro, pouco tóxicos e sua ação não é prejudicada pela presença de matéria orgânica. São recomendados para uso em pisos e pedilúvios e tem pouca atividade residual (KUANA, 2009; RUI, et. al., 2011), podendo ser aproveitados para descontaminação do ar ambiente quando utilizados na forma de fumígenos. As vantagens de se utilizar os fenóis é que de maneira

geral, os desinfetantes fenólicos quando depositados na superfície reagem com a umidade e passam a exercer ação antimicrobiana residual (PAULINO, 2006).

Dessa maneira, devido à importância e a grande variedade existe de produtos sanitizantes, é essencial que haja uma regulamentação, que garanta a qualidade desses produtos através da avaliação de parâmetros relacionados à eficácia, a segurança da aplicação e a garantia da qualidade desses produtos (ANDRADE et al., 2007).

3.3 LEGISLAÇÃO DE SANITIZANTES COM ATIVIDADE ANTIMICROBIANA

Um dos princípios básicos da produção de alimentos são as regulamentações de produtos para garantir a saúde do consumidor. No caso dos produtos de limpeza e sanitização com uso de produtos químicos, devido alguns possuírem toxicidade residual, os operadores das empresas precisam cumprir alguns objetivos, como: limitar a exposição do consumidor final aos vestígios de substâncias ativas dos produtos desinfetantes e saneantes; garantir a segurança microbiológica com uso de ferramentas capazes de controlar os organismos até à medida que eles não possam causar danos à saúde humana ou animal (GFSI, 2019).

No Brasil, os sanitizantes estão submetidos às ações de Vigilância Sanitária através da Lei nº 6.360, de 23 de setembro de 1976 (BRASIL, 1976), regulamentada pelo Decreto nº 79.094, de 05 de janeiro de 1977 (BRASIL, 1977), que dispõe sobre normas de vigilância sanitária, onde os sanitizantes são enquadrados como produtos que devem ser fiscalizados pela Vigilância Sanitária.

Além dessas leis de âmbito geral, existem legislações específicas, cujas exigências devem ser atendidas:

- RDC n° 59, de 17 de dezembro de 2010 (BRASIL, 2010) que estabelece os procedimentos referentes ao registro de produtos saneantes domissanitários levando-se em conta a avaliação e o gerenciamento de risco, considerando parâmetros como a toxicidade e a finalidade de uso do produto.
- RDC nº 14, de 28 de fevereiro de 2007 (BRASIL, 2007) classifica os sanitizantes por âmbito de aplicação, compreendendo uso geral, uso

em indústria alimentícia e afim, uso hospitalar e uso específico (BRASIL, 2007).

Os produtos com ação antimicrobiana para indústrias alimentícias e afins abrangem aqueles para uso em objetos, equipamentos e superfícies inanimadas e ambientes onde se dá o preparo, consumo e estocagem dos gêneros alimentícios, utilizados em cozinhas, indústrias alimentícias, laticínios, frigoríficos, restaurantes e demais locais produtores ou manipuladores de alimentos (BRASIL, 2007).

3.3.1 Comprovação da eficácia antimicrobiana

Em relação à comprovação da eficácia dos produtos líquidos com atividade antimicrobiana, as RDCs nº 14 e 35 afirmam que os produtos somente serão registrados e autorizados para uso após a comprovação de sua eficácia aos fins propostos, que deve ser realizada através de análise prévia com o produto acabado e nas diluições de uso indicadas pelo fabricante no rótulo. Essas análises podem ser realizadas no Instituto Nacional de Controle de Qualidade em Saúde (INCQS) da Fundação Oswaldo Cruz (Fiocruz), Ministério da Saúde, ou em laboratórios oficiais credenciados especificamente para este fim, obedecidos os métodos da *Association of Official Analytical Chemists* (AOAC) ou métodos adotados pelo Comitê Europeu de Normalização (CEN) (PINHEIRO, 2012).

Já para os saneantes de dispersão por via aérea, a legislação brasileira ainda não preconiza análises de comprovação de eficácia, contudo, conforme citado anteriomente todo o produto antimicrobiano para ter seu registro oficial para uso no território brasileiro deve ter a sua comprovação. A única normativa encontrada para essa finalidade é a Norma Francesa NF-T-72281 que apresenta a metodologia para avaliação da eficácia de agentes geradores de fumaça.

3.3.1.1 Testes do Comitê Europeu de Normatização (CEN) e testes da Norma Francesa (NF-T-72281)

O método adotado pelo CEN para a avaliação da atividade antimicrobiana de saneantes usados em ambientes domiciliares ou institucionais é composto por algumas etapas e fases. Ao longo desses testes, os produtos são avaliados quanto

à capacidade de eliminar os micro-organismos em suspensão, na presença de substâncias interferentes e em superfície (com a utilização de discos de aço inox).

A fase 1 (teste em suspensão) tem como objetivo a obtenção de um resultado preliminar sobre o produto teste, etapa utilizada principalmente pelas próprias indústrias produtoras para ajuste da concentração do princípio ativo e maior conhecimento da eficácia e características do produto que está sendo desenvolvido.

A fase 2 é a principal para testes mais completos *in vitro* e é dividida em duas etapas:

- Etapa 1 (teste em suspensão), que difere da fase 1 pela adição de substância interferente, utilizada para simular a matéria orgânica presente no ambiente, aproximando da realidade do processo de sanitização.
- Etapa 2, é um teste em superfície, com a utilização dos discos de aço inox, com o objetivo de avaliar o comportamento e eficácia do produto em uma superfície semelhante aquela onde poderá ser utilizado.

Segundo a norma do CEN, para uma análise oficial de avaliação da atividade antimicrobiana são necessárias apenas a fase 2, etapa 1 e 2 (EUROPEAN STANDARD 13687, 2001).

Assim como o método adotado pelo CEN a NF utiliza uma metodologia muito semelhante a etapa 2, teste em superfície com o uso de discos de aço inox, com peculiaridades em relação ao ambiente de realização do teste (tamanho da sala) delimitando a distância de acionamento do produto, posição dos discos, entre outros. A metodologia de cada método será mais detalhada a seguir no material e métodos.

Os fungos considerados como padrões para a realização desses testes tanto do CEN para saneantes líquidos quanto para a NF de saneantes difundidos por via aérea são as cepas de *C. albicans* ATCC 10231 e *A. brasiliensis* ATCC 16404.

4 MATERIAIS E MÉTODOS

4.1 MICRO-ORGANISMOS UTILIZADOS E PADRONIZAÇÃO DO INÓCULO INICIAL

Os fungos utilizados neste estudo estão listados na Tabela 1 e foram isolados de produtos de panificação, queijos e produtos cárneos mofados e foram identificados de acordo com o manual para identificação de espécies de *Penicillium* de acordo com Frisvad e Samson (2004) e Pitt (2000); *Aspergillus* e *Hyphopichia* foram identificados de acordo com Pitt e Hocking (2009). Diferentes cepas foram testadas dentro da mesma espécie, a fim de verificar as variações existentes na suscetibilidade dos isolados aos sanitizantes testados. Além dessas, foram utilizadas cepas comerciais adquiridas junto a um banco de cultura

Tabela 1: Cepas fúngicas utilizadas no estudo.

Fungo	**Cepa**	**Fonte de isolamento**
Aspergillus pseudoglaucus	ER 04	Panetone deteriorado, Brasil
Aspergillus pseudoglaucus	ER 05	Panetone deteriorado, Brasil
Hyphopichia burtonii	HB 100	Pão deteriorado, Brasil
Hyphopichia burtonii	HB 17	Pão deteriorado, Brasil
Hyphopichia burtonii	HB 08	Pão deteriorado, Brasil
Penicillium paneum	LMQA 03	Pão deteriorado, Brasil
Penicillium paneum	LMQA 04	Pão deteriorado, Brasil
Penicillium paneum	LMQA 05	Pão deteriorado, Brasil
Penicillium roqueforti	PR 67	Pão deteriorado, Brasil
Penicillium roqueforti	PR 06	Pão deteriorado, Brasil
Penicillium roqueforti	PR 11	Pão deteriorado, Brasil
Penicillium commune	PC 04	Queijo mussarela deteriorado, Brasil
Penicillium commune	NGT 16/12	Empanado de frango deteriorado, Brasil
Penicillium commune	CCT 7683	Ambiente, Brasil
Penicillium roqueforti	PR 02	Queijo mussarela deteriorado, Brasil
Penicillium roqueforti	PR 03	Queijo mussarela deteriorado, Brasil
Penicillium roqueforti	IMI 217568	Queijo tipo Stilton, UK
Aspergillus westerdijkiae	AW 01	Salame deteriorado, Brasil
Aspergillus westerdijkiae	AW 02	Salame deteriorado, Brasil
Aspergillus westerdijkiae	AW 03	Salame deteriorado, Brasil
Penicillium polonicum	PP 02	Presunto curado deteriorado, Brasil
Penicillium polonicum	NGT 23/12	Empanado de frango deteriorado, Brasil
Aspergillus pseudoglaucus	ER 01	Carne curada deteriorada, Brasil
Aspergillus pseudoglaucus	ER 03	Carne curada deteriorada, Brasil
Aspergillus chevalieri	IMI 211382	Sementes de café, Brasil
Aspergillus flavus	ATCC 9643	Sola de sapato, Nova Guiné
Cladosporium cladosporioides	IMI 178517	Emulsão de PVA, UK
Lichtheima corymbifera	CCT 4485	Ração, Brasil
Mucor hiemalis	CCT 4561	Solo, Brasil
Aspergillus brasiliensis	ATCC 16404	Mirtilo, USA
Candida albicans	ATCC 10231	Broncomicose, desconhecido

Fonte: o autor

Para preparar o inóculo inicial, tubos contendo Ágar Extrato de Malte (MEAc) [glicose, 20g (Neon, São Paulo, Brasil); peptona, 1g (Himedia, Mumbai, Índia); extrato de malte, 30g (Bacto^{TM}, MD, EUA); solução de traços, 1 mL; água destilada, 1L], foram inoculadas com cada cepa fúngica, seguida de incubação por 7 dias a 25 °C. Os esporos foram recolhidos por raspagem do micélio utilizando uma solução aquosa estéril de Tween 80 (0,05%). As diluições foram feitas em água peptona a 0,1% [peptona, 0,1g (Himedia, Mumbai, Índia); água destilada, 1L]. A concentração de esporos foi padronizada com auxílio de câmara de Neubauer em 10^8 esporos/mL, para testes com o agente fumigante e 10^7 esporos/mL para os demais sanitizantes. As contagens de fungos foram confirmadas por inoculação em placas de MEA e incubação durante 5 dias a 25 °C.

4.2 SANITIZANTES, CONCENTRAÇÕES E SOLUÇÕES NEUTRALIZANTES

Foram testados cinco sanitizantes químicos diferentes que foram escolhidos por conveniência entre os princípios saneantes disponíveis no mercado brasileiro e com uso autorizado na indústria alimentícia pela Agência Nacional de Vigilância Sanitária (ANVISA). Cloreto de benzalcônio (0,3%, 2,5%, 5%), biguanida (0,3%, 2,5%, 5%), ácido peracético (0,15%, 1,5%, 3%), quaternário de amônia (0,3%, 2,5%, 5%) hipoclorito de sódio [0,01% (100 ppm); 0,1% (1000 ppm); 0,2% (2000 ppm)], e um agente gerador de fumaça à base de ortofenilfenol a 15% (1g/m^3).

Os valores de concentração testados foram os valores mínimo e máximo sugeridos no rótulo dos sanitizantes, além de uma concentração intermediária, calculada a partir da média dos valores especificados no rótulo e, além disso, também foram testadas posteriormente as concentrações de hipoclorito de sódio [(0,5 %; 5000 ppm), 1,0%; 10000 ppm)]. Para o teste com o desinfetante do gerador de fumaça, foi utilizada a concentração estabelecida pelo fabricante.

Esses saneantes químicos também são os mesmos que são normalmente permitidos por outras agências internacionais, como a União Européia e os Estados Unidos (JEFFREY, 1995; WHITE, 1999; CDC, 2008).

Para garantir que a ação sanitizante ocorresse somente durante o tempo de contato do teste, foi realizada uma etapa de neutralização do agente químico. Para cada princípio sanitizante, foram utilizadas soluções desinibidoras neutralizantes

indicadas na literatura (JAENISCH et al., 2010; NORME FRANÇAISE, 2014) (Tabela 2).

Tabela 2: Sanitizantes testados, concentrações de uso recomendadas pelo fabricante e respectivos desinibidores.

Sanitizante	Princípio ativo	Concentração recomendada	Neutralizante
Cloreto de benzalcônio	Cloreto de benzalcônio	0,3 a 5%	Caldo nutriente contendo 0,5% de Tween 80 e triptona a 1%
Biguanida	Hexametileno de biguanida	2 a 5%	Caldo nutriente contendo 0,5% de Tween 80 e triptona a 1%
Ácido peracético	Ácido peracético, peróxido de hidrogênio e água	0,15 a 3%	Caldo nutriente contendo 0,6% de tiossulfato de sódio
Quaternário de amônia	Quaternário de amônia, tensoativo e água	0,3 a 5%	Caldo nutriente contendo 0,5% de Tween 80 e triptona a 1%
Hipoclorito de sódio	Hipoclorito de sódio contendo 10 a 12% de cloro ativo	0,01 a 1%	Caldo nutriente contendo 0,6% de tiossulfato de sódio
Ortofenilfenol fumigante	Ortofenilfenol 15%	1g/m^3	Caldo nutriente contendo 0,5% de Tween 80 e triptona a 1%

Fonte: o autor

4.3 EFICÁCIA *IN VITRO* DE SANITIZANTES QUÍMICOS

4.3.1 Eficácia de sanitizantes líquidos

Os testes foram realizados de acordo com os padrões estabelecidos para testes antimicrobianos de sanitizantes químicos pelo Comitê Europeu de Normalização (CEN) com adaptações (EUROPEN STANDARD 13697, 2001).

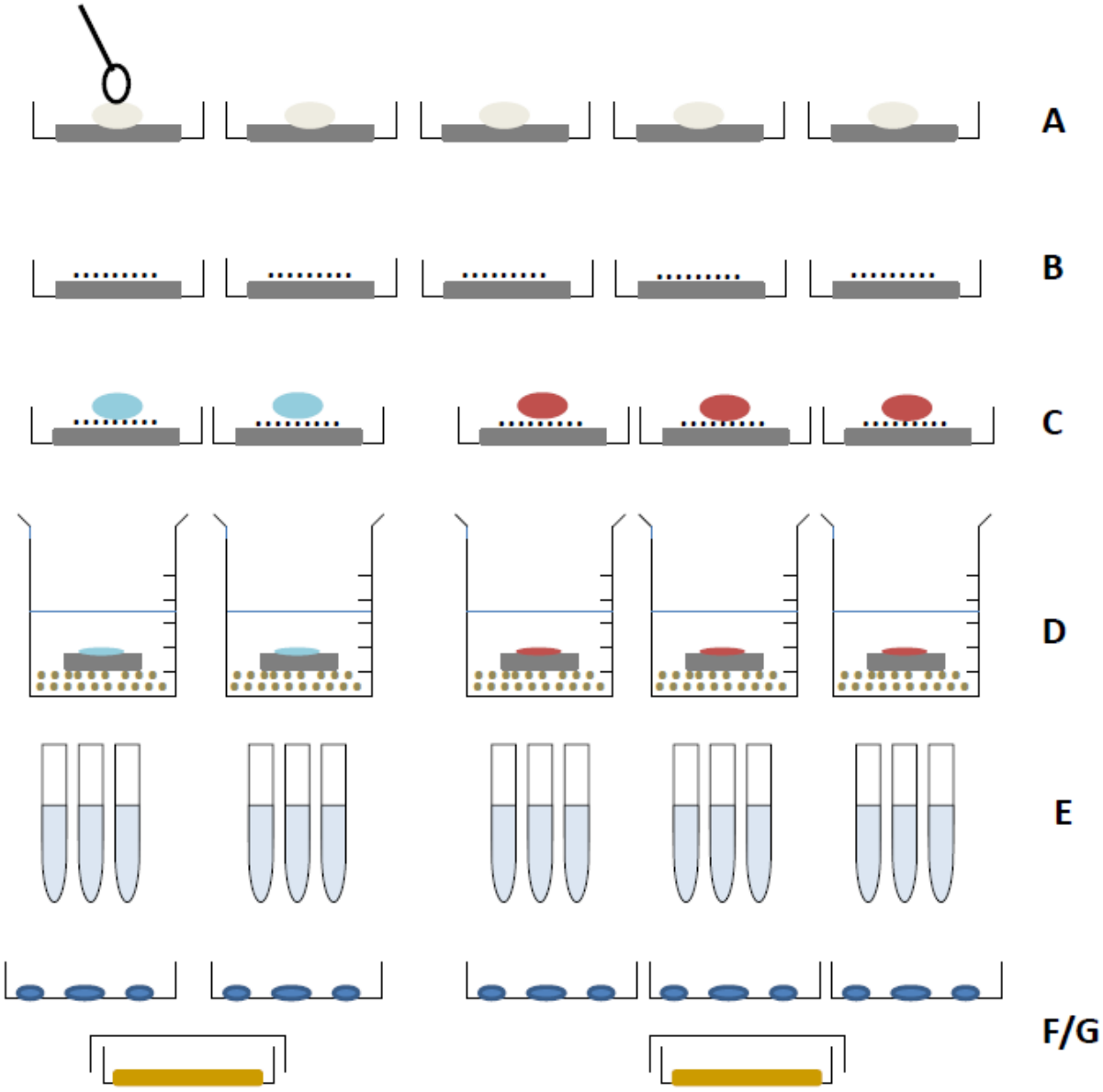
Como suporte dos micro-organismos, foram utilizado discos de aço inoxidável 304 com 2 cm de diâmetro (TSM laser®, Santa Maria, Brasil).

O teste de eficácia foi realizado a partir da contaminação de 5 discos (3 discos para o teste de sensibilidade efetiva e 2 discos para controle positivo) com 50 µL da suspensão de esporos fúngicos ajustada em 10^7 esporos/mL. Posteriorme 36 adicionado de 0,05% de leite em pó desnatado reconstituído (Elegê, São Paulo, Brasil) para simular a presença de matéria orgânica no ambiente industrial. Após a inoculação, os discos foram colocados em estufa a 35 ºC por aproximadamente 40 minutos para secagem e fixação do inóculo.

O teste de sensibilidade das espécies fúngicas foi realizado pela adição de 100 µL dos sanitizantes a cada disco contendo o inóculo fúngico seco nas três diferentes concentrações utilizadas no teste. Para avaliar o controle positivo, o desinfetante foi substituído por 100 µL de água estéril. Após 15 min de ação, os discos foram submersos em 10 mL da solução neutralizante específica por 5 min. As diluições em série foram efetuadas em 0,1% de água de peptona (10^{-1}, 10^{-2}, 10^{-3}), seguido por inoculação de uma alíquota de 1 mL em placas de Petri estéreis e misturado com 20 mL de Agar Extrato de Malte (MEA) (extrato de malte, grau alimentício, 30 g/L, ágar 15 g/L) (semeadura em profundidade). As placas foram incubadas a 25 °C durante 5 dias e posteriomente realizada as contagens das colônias e os resultados foram expressos em unidades logarítmicas (log). Todos os testes foram realizados em triplicata.

O esquema de realização do teste está ilustrado na Figura 1.

Figura 1: Esquema de realização de testes de eficácia in vitro de sanitizantes de acordo com o CEN.



A: Carreadores inoculados com 50 µL da solução de esporos ajustada em 10^7 esporos/mL + adição de leite em pó desnatado reconstituído à 0,05%.
B: Secagem em estufa a 35°C por aproximadamente 40 min para secagem e adesão do inóculo ao carreador.
C: Adição de 100 µL de água estéril (azul), para o controle positivo e inoculação de 100 µL de sanitizante (vermelho) para o teste propriamente dito, sobre o inóculo seco pelo tempo de 15 minutos.
D: Estabilização dos micro-organismos em solução neutralizante por 5 minutos seguido de agitação por 1 minuto para desprendimento e recuperação das células do disco com o auxílio de pérolas de vidro.
E: Realização de diluições seriadas.
F/G: Inoculação de 1 mL das diluições em placas de Petri seguida pela adição de meio de cultura (semeadura em profundidade), homogenização e incubação.

Fonte: o autor

4.3.2 Eficácia de sanitizante por dispersão em via aérea

Os testes foram realizados em sala fechada (~32 m³) exclusivamente utilizada e preparada para este teste. O produto testado foi um desinfetante fumigante a base de ortofenilfenol a 15% p/p.

Este produto é comercializado no Brasil e autorizado a higienizar ambientes da indústria de alimentos sem a presença de alimentos ou matérias-primas. O produto é apresentado em um formato cilíndrico de embalagem de estanho.

O acendimento do pavio do produto foi feito descolando e removendo o selo do lacre. Depois, acendeu-se o pavio e a fumaça se dispersou imediatamente. O sanitizante foi aceso no chão de acordo com as instruções do manual do fabricante. O fabricante indica uma concentração de OPP de 1g de produto por m³ e tempo de exposição de 7h (o local da aplicação deve permanecer fechado por 7h e, uma vez aberto, o ambiente deve ser ventilado por pelo menos 15 min antes da entrada de pessoas). Também é recomendável limpar superfícies e equipamentos antes de reutilizá-los.

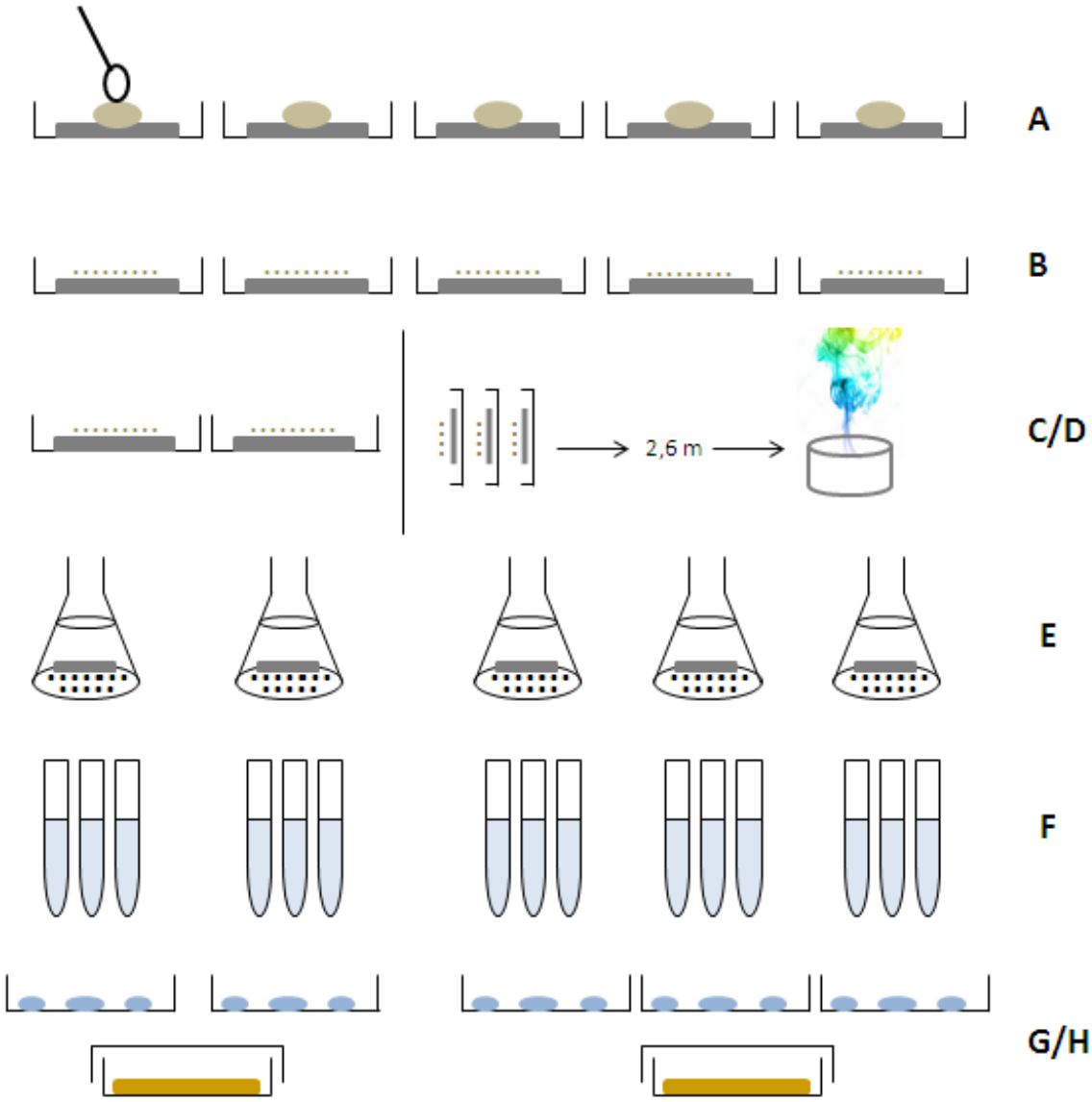
O teste de eficácia do fumígeno foi realizado de acordo com a NF-T-72281, com adaptações (com adaptação no valor da concentração inicial de esporos para os testes, de 10^5 esporos/mL como preconiza a norma, para 10^8 esporos/mL, a fim de visualizar de forma mais segura a real eficácia antifúngica dos produtos) (Figura 2). Discos de aço inox de 2 cm de diâmetro foram inoculados com 50 µL de uma suspensão de esporos fúngicos previamente ajustados a 10^8 esporos/mL e adição de 0,05% de leite em pó desnatado reconstituído (Elegê, São Paulo, Brasil) para simular a presença de matéria orgânica. Cinco discos foram inoculados com os fungos a serem testados em cada experimento. Três discos foram expostos ao agente e usados para testar a ação do desinfetante. Os outros dois discos não foram expostos ao desinfetante e utilizados como controle positivo do inóculo fúngico.

Uma vez inoculados, os discos de ambos os testes foram colocados no forno a 35 °C por aproximadamente 40 min para fixar o inóculo. Após esse período, os discos do controle positivo foram mantidos no laboratório durante o mesmo período em que os outros discos foram expostos ao desinfetante (7 horas).

A exposição ao agente fumigante foi feita colocando os discos a 2,6 m de distância do ponto onde o sanitizante foi liberado. Os discos foram posicionados

verticalmente com a superfície contendo o inóculo voltado para o lado oposto da fumigação.

Figura 2: Esquema de realização de testes de eficácia in vitro de sanitizantes de acordo com a NF-T-72281.



A: Carreadores inoculados com 50 µL da solução de esporos ajustada em 10^7 esporos/mL + adição de leite em pó desnatado reconstituído a 0,05%.
B: Secagem em estufa a 35°C por aproximadamente 40 min para secagem e adesão do inóculo ao carreador.
C/D: Discos expostos a fumaça por 7 horas.
E: Estabilização dos micro-organismos em solução neutralizante por 5 minutos seguido de agitação por 1 minuto para desprendimento e recuperação das células do disco com o auxílio de pérolas de vidro.
F: Realização de diluições seriadas, se necessidade.
G/H: Inoculação de 1 mL das diluições em placas de Petri seguida pela adição de meio de cultura (semeadura em profundidade), homogenização e incubação.

Fonte: o autor

No final do tratamento, ambos os carreadores inoculados (não expostos e expostos ao agente fumigante) tiveram os micro-organismos viáveis recuperados em 100 ml de líquido de recuperação e agitados durante 1 min com 10 g de pérolas de vidro. Diluições seriadas (10^{-1}; 10^{-2}; 10^{-3}) foram realizadas em água peptona a 0,1% (m/v).

Uma alíquota de 1 mL de cada diluição foi adicionada a placas de Petri estéreis. Em seguida, adicionaram-se 20 ml de Agar Extrato de Malte (MEA) (extrato de malte, grau alimentício, 30 g/L, 15 g/L de agar), homogeneizou-se e deixou-se solidificar (semeadura em profundidade). As placas foram incubadas a 25 °C durante 5 dias, as colânias foram então contadas e os resultados expressos em unidades logarítmicas (log). Todos os testes foram realizados duas vezes e em dias diferentes.

4.4 ANÁLISE DOS DADOS

4.4.1 Análise estatística

A eficácia de cada desinfetante foi avaliada pela diferença entre o número de células fúngicas recuperadas do controle positivo (sem exposição ao sanitizante) e os micro-organismos expostos ao sanitizante.

De acordo com o padrão do CEN, o sanitizante deve reduzir, no caso de fungos, 3 log (99,9%) do número inicial de micro-organismos recuperados no controle positivo para ser considerado um sanitizante eficaz (EUROPEAN STANDARD 13697, 2001).

De acordo com a NF-T-72281, para ser considerado um desinfetante efetivo, o agente fumigante deve ser capaz de reduzir 4 log (99,99%) do número inicial de micro-organismos na população de fungos testada (expostos a fumigação) quando comparados aos recuperados no controle positivo.

Uma análise de variância (ANOVA) foi realizada. Os dados de recuperação dos fungos após a exposição aos sanitizantes comerciais foram analisados pelo teste de Scott-Knott ($p < 0,05$). As análises estatísticas foram realizadas utilizando a versão 5.6 do software SISVAR® (FERREIRA, 2011).

4.4.2 Escala de eficácia

A partir dos resultados obtidos foi elaborada uma escala de 5 pontos a fim de avaliar a eficácia dos sanitizantes:

• máxima eficácia = quando o agente reduz mais de 4 log de fungos em relação ao controle positivo;

• boa eficácia = quando a redução fúngica é entre 3,9 e 3 log;

• eficácia reduzida = quando a redução fúngica é entre 2,9 e 2 log;

• baixa eficácia = quando a redução fúngica é entre 1,9 e 1 log;

• ineficaz ou sem efeito = quando a população inicial permanece inalterada.

5 ARTIGOS CIENTÍFICOS INTEGRADOS

5.1 ARTIGO 1 – EFFICACY OF COMMERCIAL SANITIZERS AGAINST FUNGI OF CONCERN IN THE FOOD INDUSTRY

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Efficacy of commercial sanitizers against fungi of concern in the food industry

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ABSTRACT

Choosing an active ingredient for sanitizing agents, and the concentrations to be employed for hygiene in food industries is an important step in order to obtain the maximum efficacy of a particular product. However, the data available on the sensitivity of food spoilage fungi to sanitizers are very limited. Thus, the aim of this work was to evaluate the antifungal efficacy of commercial sanitizing agents appropriate for use in food industries on species with potential for food spoilage. Tests were carried out following a European Committee for Standardization (CEN) protocol for testing the antifungal effect of chemical sanitizers, with adaptations. Six fungi were used: *Aspergillus brasiliensis* (ATCC 16404), *Candida albicans* (ATCC 10231), *Cladosporium cladosporioides* (IMI 158517), *Penicillium commune* (CCT 7683), *P. polonicum* (NGT 33/12) and *P. roqueforti* (IMI 217568); five sanitizers were used at three different 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 (0.1%, 0.5%, 1.5%). Variation in the antifungal efficacy of sanitizers was seen. Sodium hypochlorite was the most efficient agent at the concentrations evaluated, and biguanide should not be chosen for controlling food spoilage fungi.

1. Introduction

The deterioration of food by filamentous fungi starts with contamination of the product by fungal spores originating from the environment. When intrinsic parameters, such as water activity (a_w) and pH, as well as temperature, are favorable, the spores will germinate and form a visible mycelium, deteriorating the product (Dagnas & Membré, 2013; Gougouli, Kalantzi, Beletsiotis, & Koutsoumanis, 2011). Spoilage by fungi is more common in breads, cheeses, fruits and vegetables. It occurs because, even though they may have a bacterial load, these products have parameters restrictive to growth of most of these bacteria, but not strong enough to avoid the multiplication of spoilage fungi. This problem occurs during the cooling, maturation and packing stages, in the case of cheeses; storage, in the case of fruits and vegetables; and baking, in the case of breads (Decontardi, Mauro, Lima, & Battilani, 2017; Garcia & Copetti, 2018; Jay, 2005; Leggieri, Decontardi, & Battilani, 2018; Muhammad, Shehu, & Amusa, 2004; Ropars, Cruaud, Lacoste, & Dupont, 2012).

In the context of the deterioration caused by fungi in these types of food products, the genera *Aspergillus*, *Penicillium*, *Cladosporium* and *Candida* are highlighted (Pitt & Hocking, 2009). The main source of food-deteriorating fungi is microbial cells dispersed as propagules in the processing environment (Hedrick & Heldman, 1969). It includes air, surfaces and processing equipment, as well as raw materials (Kure, Skaar, & Brendehag, 2004; Vacheyrou et al., 2011).

Reducing microbial contamination of the production environment is one of the most effective ways to minimize spoilage of a food product, increasing its shelf life. Adoption of hygienic-sanitary measures, such as the application of an effective cleaning method followed by a sanitization process using appropriate sanitizing agents at adequate concentrations, is recommended to achieve the objectives (Kuaye, 2017; Rutala, 1996).

Even then, the success of disinfection depends not only on the characteristics of the product and the way it is used, but also on the characteristics of the microorganisms present in the site and their sensitivity to the sanitizers employed. Knowledge of the fungal sensitivity to each class of sanitizing agent appropriate for use in food industries would be extremely useful to guide hygiene processes, improving the efficacy of sanitation. However, the results available in the literature are very limited, and the methodologies used are quite diverse, generally not following a standardized official protocol.

To reduce this gap, the objective of this work was to evaluate the

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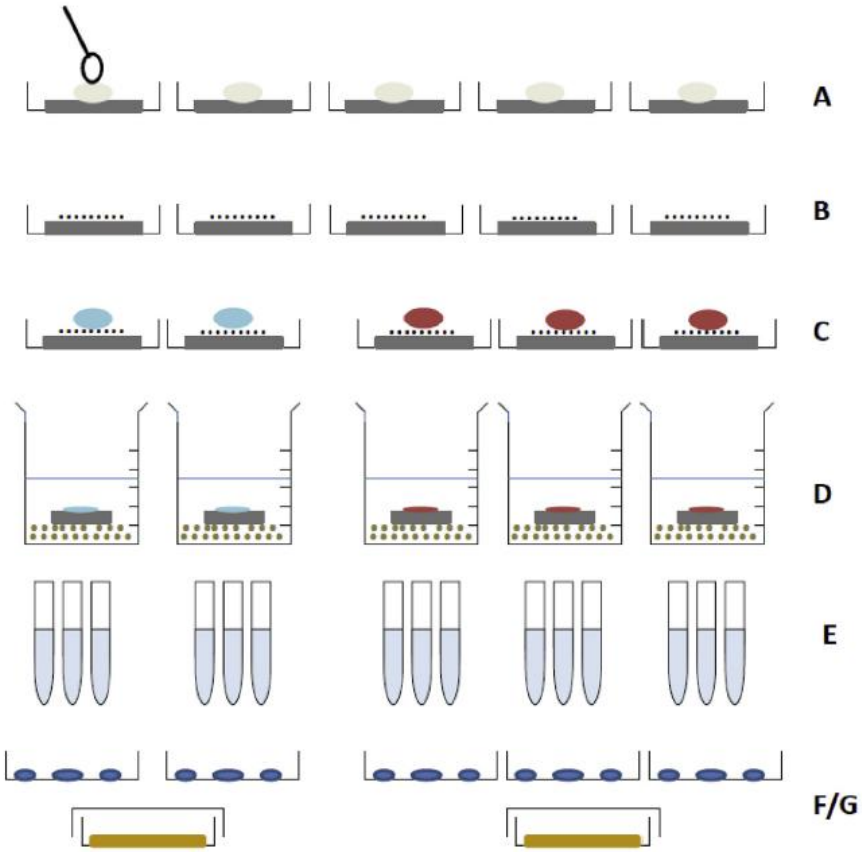
Table 1
Sanitizers with permitted use in food production environments tested, concentrations recommended by manufacturers for use and respective neutralizers.

SANITIZER	ACTIVE PRINCIPLE	RECOMMENDED CONCENTRATION	NEUTRALIZING
Benzalkonium chloride	Benzalkonium chloride	0.3–5%	Nutrient broth with 0.5% of tween 80 and triptone 1%
Biguanide	Hexamethylene biguanide hydrochloride	2–5%	Nutrient broth with 0.5% of tween 80 and triptone 1%
Peracetic acid	Peracetic acid, hydrogen peroxide, Acetic acid	0.15–3%	Nutrient broth with 0,6% of sodium thiosulphate
Quaternary ammonia	Quaternary ammonia, tensoative and water.	0.3–5%	Nutrient broth with 0.5% of tween 80 and triptone 1%
Sodium hypochlorite	Sodium hypochlorite 10–12% of active chlorine	0.1–1%	Nutrient broth with 0.6% of sodium thiosulphate



A: Carriers inoculated with 50 µL of spore solution adjusted to 108 spores / mL + addition of reconstituted powdered milk 0.05%.
B: Greenhouse drying at 35 ° C for approximately 40 min for adherence of the inoculum in the carriers.
C: Addition of 100 µL sterile water (blue), to carry out the positive control and inoculation of 100 µL sanitizer (red) for the test itself, on the dry inoculum for the time of 15 minutes.
D: Stabilization of the microorganisms in neutralizing solution for 5 minutes followed by shaking for 1 minute for detachment and recovery of the disc cells with the aid of glass beads.
E: Perform serial dilutions as needed.
F/G: Inoculation of 1 mL of the dilutions in Petri dishes followed by addition of culture medium (deep seeding), homogenization and incubation (deep seeding).

Fig. 1. Scheme for *in vitro* testing antifungal efficacy of sanitizers according the standard established by the European Committee for Standardization (CEN) (European Standard 13697, 2001).

antifungal efficacy of commercial sanitizing agents appropriate for use in food industries against the main food spoilage fungi.

2. Materials and methods

2.1. Sanitizers, recommended concentrations and neutralization

Five different sanitizers (Table 1) authorized for use in food industries by resolution RDC no. 14/2007 of the Brazilian National Sanitary Surveillance Agency (ANVISA) were selected. These principles are usually allowed worldwide for food industry sanitization, including authorization by EU and USA sanitary authorities (CDC, 2008; EPA, 1999; Jeffrey, 1995).

The minimum and maximum values tested were those recommended by the manufacturers on the sanitizer labels. Additionally, an intermediate concentration was calculated from the mean of these values (Table 1).

To ensure that the efficacy of each sanitizer occurred only during the contact time of the test, neutralization was performed using the neutralizers indicated in the literature for each sanitizing principle evaluated in this study (Jaenisch, Kuchiishi, & Coldebella, 2010) (Table 1).

2.2. Microorganisms used and standardization of the initial inoculum

Six strains were used, two standards for sanitization tests: *Aspergillus brasiliensis* (ATCC 16404) and *Candida albicans* (ATCC 10231), and four strains commonly involved in the spoilage of food products: *Cladosporium cladosporioides* (IMI 158517), *Penicillium commune* (CCT 7683), *P. polonicum* (NGT 33/12) and *P. roqueforti* (IMI 217568).

All strains were lyophilized and kept under refrigeration (7 ± 1 °C) for preservation of microbial characteristics and quality assurance of viable cells during the period of the experiment.

For preparation of the initial inoculum, each strain was inoculated in tubes containing malt extract agar (MEA) [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] and incubated 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.

2.3. In vitro antifungal efficacy of commercial sanitizers

Tests were carried out following the standards established by the European Committee for Standardization (CEN) (European Standard 13697, 2001) for disinfectant testing using carriers for bactericidal and fungicidal efficacy tests, with adaptations. The scheme of the assay performed is illustrated in Fig. 1.

According to the CEN standard, the effectiveness of a sanitizer is expressed by the logarithmic reduction in colonies obtained between the positive control and the tested population exposed to the product. To be considered as efficient sanitizing agent, it should reduce the initial amount of fungal colony-forming units recovered from the positive control by 3 log units (European Standard 13697, 2001). In this study, the effectiveness of a sanitizer was evaluated by counting, after 5 days of incubation as recommended fungal enumeration by the International Commission on Food Mycology (Hocking, Pitt, Samson, & Thrane, 2006), the colonies formed after fungal germination and mycelium growth.

As carriers of the microorganisms, 304 stainless steel discs of 2 cm diameter, previously treated with 5% Triton™ X-100 (Sigma-Aldrich, USA) for 60 min to remove impurities, were used. The discs were rinsed and left immersed in 70% (v/v) isopropanol (Neon, São Paulo, Brazil) for 24 h. After that, they were dried and stored in a sterile vial until being used in the test.

The test was performed by the contamination of five discs with 50 µL of the fungal spore suspension adjusted to 10^8 spores/mL followed by addition of 0.05% reconstituted skim milk powder (Elegê, São Paulo, Brazil), simulating the presence of organic matter present in the environment. For each fungal species evaluated, three discs were used to test the disinfectant efficacy (effective sensitivity), and the other two discs were used as positive controls (not exposed). After the inoculation process, both groups of discs (effective sensitivity and positive control) were taken to a bacteriological stove at 35 °C, for approximately 40 min, to fix the inoculum on the carriers.

Each sensitivity test was performed by adding 100 µL of sanitizer to a disc containing the fixed microbial inoculum, at three different concentrations [peracetic acid (0.15%, 1.5%, 3%); biguanide (2%, 3.5%, 5%); benzalkonium chloride (0.3%, 2.5%, 5%); sodium hypochlorite (0.1%, 0.5%, 1.5%); quaternary ammonium (0.3%, 2.5%, 5%)]. For evaluation of the positive control, the sanitizer was replaced with 100 µL of sterile water.

After 15 min of action, the discs were immersed for 5 min in a liquid containing 10 mL of the specific neutralizing solution for each disinfectant (Table 1). Serial dilutions in 0.1% peptone water were made. The pour plate method was used; 1 mL was inoculated into sterile Petri dishes and mixed with 20 mL of MEA (malt extract, food grade, 30 g/L, agar 15 g/L).

Plates were incubated at 25 °C for 5 days; growing colonies were counted as colony-forming units, and results were expressed in logarithmic units (log). Incubation for 5 days was chosen because the size of colonies of the fungi evaluated after that time allows for easily counting with the naked eye. The efficacy of each sanitizer was evaluated by the difference between the number of fungal colony-forming units recovered from the positive controls and that from discs exposed to sanitizer. All tests were performed under aseptic conditions and in triplicate.

2.4. Antifungal efficacy scale

Based on the results obtained, an efficacy antifungal scale was elaborated, based on the ability of the sanitizer to reduce the count of viable cells after exposure (in logarithmic cycles, log). The scale was divided into five comparative parameters: maximum efficacy (+++ +), when reducing the fungal count by at least 4 log in relation to the positive control; good efficacy (+++), when reducing the initial fungal count by 3.9 to 3 log; reduced efficacy (++), when it reduced the initial fungal count by 2.9 to 2 log; poor efficacy (+), when it reduced the initial fungal count by 1.9 to 1 log; and inefficacy or no effect (−), when the microbial population remained unchanged.

3. Results and discussion

3.1. In vitro antifungal efficacy of commercial sanitizers

Variable antifungal efficacy was observed among sanitizers. Differences were observed both among the concentrations of the same sanitizer and the fungal species exposed to the same sanitizer concentration (Fig. 2).

The variation could be due to the different modes of action of each sanitizer. Some sanitizers, such as quaternary ammonium compounds, act on the cell membrane, altering its permeability and causing cell depletion due to stimulation of glycolysis; others, such as peracetic acid, act by oxidizing the cellular components of microorganisms, damaging the enzymatic system (Nascimento, Delgado, & Barbaric, 2010). Studies indicate that the success of fungal survival is due to their structural versatility for immediate dimorphism and for diverse associations, their own characteristics of cell wall biosynthesis (Bernard &

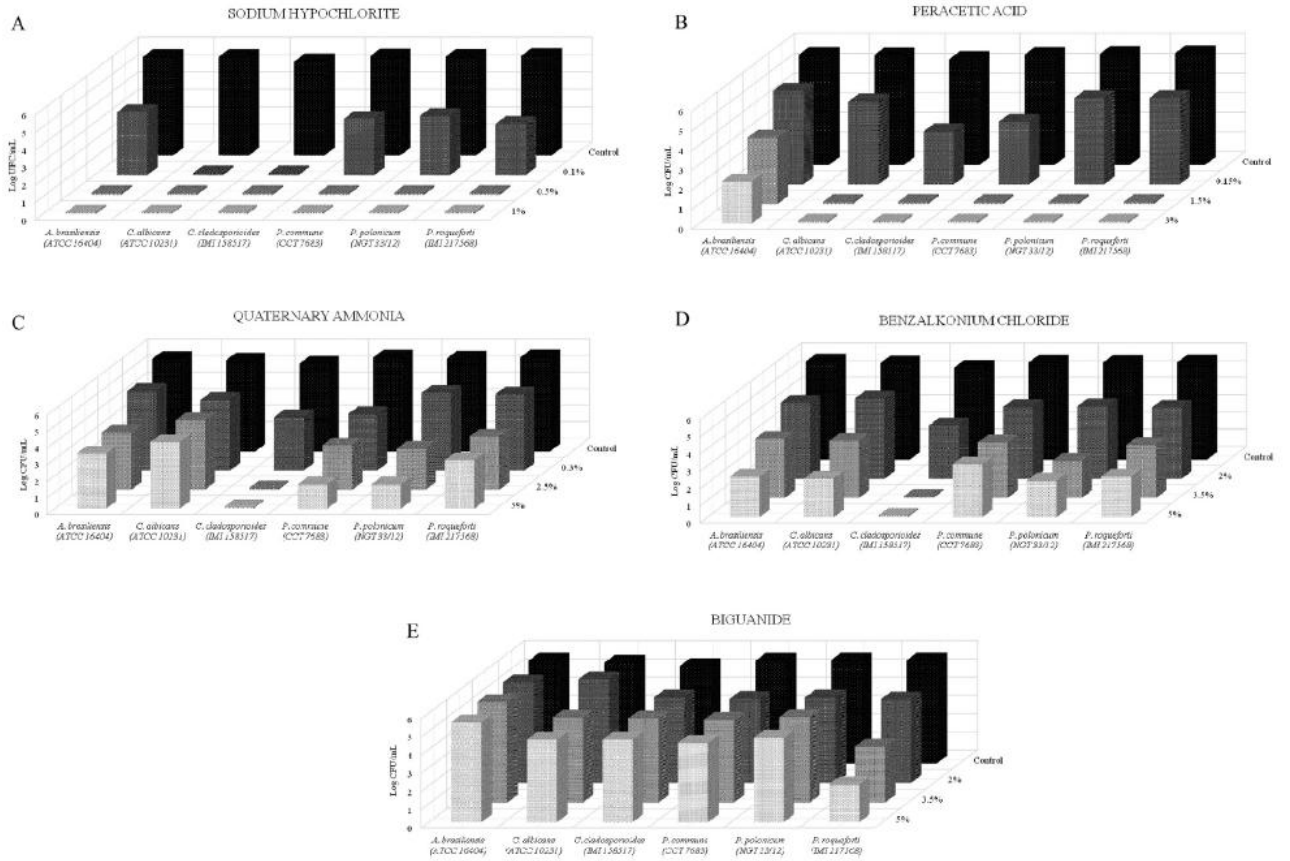


Fig. 2. Efficacy of commercial sanitizers against food spoilage fungi (*Aspergillus brasiliensis* (ATCC 16404), *Candida albicans* (ATCC 10231), *Cladosporium cladosporioides* (IMI 158517); *Penicillium commune* (CCT 7683); *Penicillium polonicum* (NGT 33/12) and *Penicillium roqueforti* (IMI 217568)) tested with the recommended use concentrations. Peracetic acid (A); Sodium hypochlorite (B); Ammonium quaternary (C); Benzalkonium chloride (D) and Biguanide (E).

Latagé, 2001; Lewis, 2001), the ability to generate biofilms, or metabolic adaptation to commercially available compounds (Sanglard, Ischer, Monod, & Bille, 1996; Shin, Kee, & Shin, 2002).

Sodium hypochlorite (Fig. 2A) was the most efficient sanitizer against the fungal species evaluated, even in the presence of organic matter (milk, in the test). Organic matter can affect the antimicrobial efficacy of sodium hypochlorite because this compound stabilizes the cytoplasmic membrane against permeability, allowing the recovery of injured bacterial cells (Virto, Mañas, Álvaerz, Condon, & Raso, 2005). Although those studies were restricted to bacteria, the occurrence of organic material in the processing environments of food industries is a reality and could also affect the antifungal effectiveness of this compound. Chlorine presents activity even at low temperatures, has low cost and remains minimally in the form of residues or films on surfaces. For these reasons, it is one of the compounds most used in sanitization processes, being considered one of the best options for food industries (Kuaye, 2017).

Sodium hypochlorite reduced by 3 log almost all species at all concentrations tested, only having limited action against *P. commune* (CCT 7683) and *P. polonicum* (NGT 33/12) at the lowest concentration tested, with a reduction of 2 log cycles. These two species of *Penicillium* sp. are commonly related to the spoilage of cheeses (Hayaloglu & Kirbag, 2007; Kure & Skaar, 2000; Kure, Skaar & Brendehag 2004; Kure, Waeteson, Brendehag & Skaar, 2001) and dry-cured fermented sausages (López-Díaz, Santos, Garcia-López & Otero, 2001; Papagianni, Ambrosiadis, & Filiousis, 2007). In addition, *P. commune* is associated with the phenolic defect that occurs during the maturation of Italian hams (Spotti, Mutti, & Campanini, 1988), and *P. polonicum* (NGT 33/12) is the main species spoiling frozen chicken nuggets (Saccomori, Wiggman, Bernardi, Alcano-González, & Copetti, 2015). In these specific food industries, if sodium hypochlorite is the sanitizer of choice, use of lower concentrations should be avoided in order to achieve the desired efficacy.

The results obtained for evaluation of the sensitivity of fungal species to sodium hypochlorite can be broadly compared to other reports from the literature. Using different methodologies, such as dilution tests and different exposure times, sodium hypochlorite inhibited *Saccharomyces cerevisiae* at 0.1% (Winniczuk & Parish, 1997) and *Aspergillus niger* at 0.2% concentration (Ozyurt, 2000). In addition, Reynolds, Bone, Bright, and Gerba (2004) achieved a reduction of more than 5 log of *Penicillium*, *Cladosporium*, *Mucor*, *Rhizopus*, *Alternaria* and *Aspergillus* after 5 min of exposure to this agent at a concentration of 2.4%.

Peracetic acid showed no action at the lowest recommended concentrations when evaluated against all the species tested, with the exception of *C. cladosporioides*. On the other hand, this agent was efficient against all species tested at the highest concentration, and *A. brasiliensis* was the only species not sensitive to the intermediate concentration tested (Fig. 2B). So, the minimum value of the wide range of concentrations used (0.15%–3%) should be revised, at least when using it for fungal control.

Peracetic acid is commonly employed for the reduction of fungal contamination of ambient air and is also widely used in the routine of industries. This product has a broad spectrum of action, rapidly inactivating microorganisms and acting through cell membrane oxidation principles, and is categorized as a toxicologically safe acid disinfectant

Table 2
Antifungal efficacy of commercial sanitizing agents appropriate for use in food industries on species with potential for food spoilage, following the proposed scale.

SANITIZER TESTED	CONCENTRATION TESTED (%)	EFFICACY AGAINST FUNGI*					
		Aspergillus brasiliensis (ATCC 16404)	*Candida albicans* (ATCC 10231)	*Cladosporium cladosporioides* (IMI 158517)	*Penicillium commune* (CCT 7683)	*Penicillium polonicum* (NGT 33/12)	*Penicillium roqueforti* (IMI 217568)
Benzalkonium chloride	2	+	+	++	+	+	+
	3.5	++	++	++++	++	+++	++
	5	+++	+++	++++	++	+++	+++
Biguanide	2	-	-	-	+	+	+
	3.5	-	-	-	+	+	++
	5	-	+	-	+	+	+++
Peracetic acid	0.15	+	+	++	++	+	+
	1.5	++	++++	++++	++++	++++	++++
	3	+++	++++	++++	++++	++++	++++
Quaternary ammonia	0.3	+	+	++	++	+	+
	2.5	++	+	++++	++	+++	++
	5	++	+	++++	++++	++++	++
Sodium hypochlorite	0.1	+++	++++	++++	++	++	++
	0.5	++++	++++	++++	++++	++++	++++
	1	++++	++++	++++	++++	++++	++++

(*) ++++ = maximum efficacy; +++ = good efficacy; ++ = reduced efficacy; + = poor efficacy; - = inefficacy or no effect.

(McDonnell, Grignol, & Antloga, 2002).

The species most sensitive to peracetic acid, *C. cladosporioides*, is extensively related to fungal spoilage of several industrialized and *in natura* products, such as fresh fruits (Muhammad et al., 2004). Is considered a psychrophilic fungus, commonly related to the spoilage of refrigerated cheeses and described as one of the main causes of the so-called "black spot" defect in cooled meats and cured hams (Alía et al., 2016; Lozano-Ojalvo et al., 2015). Since the genus has been isolated from several meat processing plants, always from air or from the product surface (from raw materials) (Sørensen, Jacobsen, Nielsen, Frisvad, & Kock, 2008), it is important to emphasize the importance of industrial environmental sanitization to prevent fungal propagules from becoming deposited in food and causing it to spoil early. In industries where *Cladosporium* sp. is the problem, both sodium hypochlorite and peracetic acid are good options, achieving good results even at low dosages.

In a study conducted by Korukluoglu, Sahan, and Yigit (2006), great efficacy of action was obtained when *A. niger* and *P. roqueforti* were exposed to peracetic acid at a concentration of 0.3% for 55 or 60 min, only allowing for broad comparisons. *A. brasiliensis* shares morphological similarities with *A. niger*. In our study, peracetic acid was only effective against *A. brasiliensis* when using the highest concentration of this sanitizer.

For quaternary ammonium and its derivative benzalkonium chloride, in general, effective action was only seen at the highest concentration (Figs. 2C and 1D); *C. cladosporioides* and *P. polonicum* were exceptions. These two species were sensitive at the intermediate concentrations of both agents. On the other hand, *A. brasiliensis* (ATCC 16404) and *C. albicans* (ATCC 10231) were resistant to quaternary ammonium at all concentrations tested.

A. brasiliensis (ATCC 16404) and *C. albicans* (ATCC 10231) are the standard strains for antifungal efficacy assays of sanitizers. This result demonstrates the importance of making individualized evaluations for efficacy of the compounds against the problem microorganisms present in each food industry. Although quaternary ammonium was inefficient against the standard strains, it demonstrated efficacy at the intermediate concentrations used, higher for half of the species tested. On the other hand, even though benzalkonium chloride was efficient at the highest concentration against the standard strains, it was inefficient against *P. commune*.

Based on the results obtained, quaternary ammonium and benzalkonium chloride should not be the sanitizers of choice for cheese industries, due to their limited efficacy of action against *P. commune* and *P. roqueforti*.

The observed resistance of *P. roqueforti* (IMI 217568) to quaternary ammonium was also reported by Bungaard-Nielsen and Nielsen (1995) in a test against *P. roqueforti* (IBT 11524) and *P. carneum* (IBT 14042). Conversely, in a study conducted by Korukluoglu et al. (2006), *P. roqueforti* was sensitive to this sanitizing agent at a concentration of 2% after 2–11 min of exposure, suggesting that strains or closely related species may show variation.

Biguanide hexamethylene was the sanitizer with less efficacy against all the fungi tested (Fig. 2E) and should not be the compound of choice when the main goal of an industry is fungal control.

Polymeric biguanides are the main active component of some products widely used in sanitary decontamination of food industries, although their indication is related to hand hygiene (Avecia, 2004).

3.2. Efficacy scale

A scale was proposed to facilitate general evaluation of the antifungal efficacy of sanitizing agents appropriate for use in food industries (Table 2).

Due to the variable results obtained regarding the sensitivity of fungal species to different classes of sanitizers, and knowing that some fungal species are more related to the deterioration of a certain class of foods, each food industry should select the sanitizer to be applied by considering its efficacy against the species which is a problem for it. This knowledge is more common for bacteria, but few studies have focused on fungi, and much remains to be covered on fungal control. This perception agrees with Lorin et al. (2017), who highlighted the importance of evaluating problem microorganisms individually, not only standard strains, for this type of test, to give the industry more coherent steps for decontamination strategies.

4. Conclusion

Results were variable, so identification of the fungal spoilers present in each food factory and testing their sensitivity to the sanitizers available will help in decisions about the most adequate product and the optimal concentrations to be applied for their control.

Sodium hypochlorite was the most efficient sanitizer for the species and concentrations evaluated in this study, followed by peracetic acid. On the other hand, benzalkonium chloride and quaternary ammonium

presented variable fungal reduction. Biguanide has limited efficacy and should not be the compound of choice when the primary goal of an industry is controlling food spoilage fungi.

Conflicts of interest

The authors declare no conflict of interest.

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5.2 ARTIGO 2 – SENSITIVITY OF FOOD SPOILAGE FUNGI TO A SMOKE GENERATOR SANITIZER

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Sensitivity of food spoilage fungi to a smoke generator sanitizer

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ABSTRACT

Smoke generator sanitizers are easy to handle and can access to hard-to-reach places. They are a promising alternative for controlling food and air borne fungi, which are known to cause losses in the bakery, meat, and dairy industries. Therefore, the present study aimed to evaluate the efficiency of a smoke generator sanitizer based on orthophenylphenol against ten fungal species relevant to food spoilage. The tests were carried out according to the norms by the French protocol NF-T-72281, with adaptations specific for disinfectants diffused in the air. The tests were performed in an enclosed room of approximately 32 m^3. *Aspergillus brasiliensis* (ATCC 16404), *Candida albicans* (ATCC 10231), *Aspergillus flavus* (ATCC 9643), *Aspergillus chevalieri* (IMI 211382), *Cladosporium cladosporioides* (IMI 158517), *Lichtheima corymbifera* (CCT 4485), *Mucor hiemalis* (CCT 4561), *Penicillium commune* (CCT 7683), *Penicillium polonicum* (NGT 33/12), and *Penicillium roqueforti* (IMI 217568) were exposed to the smoke generator sanitizer for 7 h. The product was efficient against *C. albicans* and *C. cladosporioides*, although it was unable to reduce 4 log of the other tested species. The variable sensitivity of the fungal species to the sanitizer emphasizes the importance of confronting a target microorganism (causing problems in a specific food industry) with the sanitizer aiming to control it and obtain satisfactory results in hygiene programs.

1. Introduction

Fungi are widely distributed in the environment and can be found in the air, water, and soil. In order to adapt to their surroundings, these microorganisms must have different parameters of multiplication, sporulation capacity and dissemination (Pitt and Hocking, 2009). Airborne fungi are dispersed in the air in the form of small propagules and spread through airflow in the production environments of food industry facilities. This action is facilitated by the activity of collaborators, communication between distinct rooms, floor drains, and the ventilation system. These fungal particles, which are mostly spores, act as a contamination source at the location in which they settle in (Hedrick and Heldman, 1969).

After contaminating the food, the multiplication of these microorganisms can lead to product spoilage, promoting considerable economic losses, and in some cases, even pose risks to consumer health (Filtenborg et al., 1996; Pitt and Hocking, 2009; Samson et al., 2004). However, the negative economic impact can be minimized by contamination prevention and attention to the factors that enable microbial multiplication in food products (Asefa et al., 2010). Adopting hygienic-sanitary measures, such as effective cleaning and sanitization methods, can reduce the contamination of surfaces and the environment to adequate levels (Kuaye, 2017). It is well known that clean environments have reduced microbial counts and, therefore, less initial microbial load in processed foods, which extends their shelf life.

Smoke generator sanitizers are easy to handle, can access hard-to-reach places, and exert their effect during the exposure period while leaving little or no residue. These agents can be an important alternative for microbial control in food industries (Sholberg et al., 2004). In general, there is great concern to prevent bacterial development due to food safety issues. On the other hand, fungi are commonly identified just when the problem reaches considerable magnitudes, which results in food waste and economic losses (Dagnas et al., 2017; Garnier et al., 2017). Aerial dispersion of spores is considered a crucial point in controlling food spoilage fungi (Samson et al., 2004). Smoke generator agents are usually cheap and easy to use, making them a viable alternative to the bakery, meat, and dairy industries. These industries are the most affected by fungal deterioration, being air contamination a critical point for spore dissemination (Chitarra and Chitarra, 2005).

Phenolic sanitizers, such as orthophenylphenol (OPP) ($C_{12}H_{10}O$),

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are broad-spectrum, low-toxicity germicides, and their action is not impaired by the presence of organic matter. In addition to their recommended use on floors, footbaths, and having little residual activity (Grezzi, 2008), phenolic sanitizers can be used to decontaminate ambient air if used as smoke generator agents.

In Brazil, the use of orthophenylphenol-based disinfectant agents is authorized to sanitize food-processing environments and related industries. Moreover, the disinfectant tested in the present study is the first authorized in the country for this purpose (ANVISA (National Health Surveillance Agency), 2013). Orthophenylphenol-based disinfectant agents should be applied to food industry environments without the presence of food, even if no significant residual effects on citrus fruit are observed and they are below the residual limits recommended by Codex Alimentarius (10 mg Kg^{-1}) and the European Union (5 mg Kg^{-1}) (Besil et al., 2016). Orthophenylphenol exhibited low acute toxicity in animal experiments (Bomhard et al., 2002). The International Agency for Research on Cancer (IARC) classifies OPP as belonging to Group 3 (not classifiable as to its carcinogenicity to humans) (IARC (International Agency for Research on Cancer), 1999). Moreover, this organic compound is known to cause irritation to the skin, eye, and respiratory tract (PubChem, 2018), and, as a result, it should be manipulated with caution.

In countries close to Brazil, such as Uruguay, OPP or its sodium salt are used as a specific post-harvest fungicide (Besil et al., 2016). In other countries, such as the United States and European Union (EU) countries, this compound is most commonly used to decontaminate hospital areas. Some EU countries often employ this agent as a pesticide and preservative for citrus fruit and vegetables because of its efficacy as a biocide against bacteria, fungi, and yeasts (CDC, 2018; FAO, 1999; Coelhan et al., 2006).

Data on the antifungal activity of smoke generator agents against fungal species involved with food spoilage were not found in the literature. Therefore, this study aimed to verify the efficiency of an OPP-based fumigant sanitizer dispersed, in the air in the form of dry smoke, against fungal species present in the air of food industries and commonly involved in the deterioration of food products.

2. Materials and methods

2.1. Smoke generator sanitizer, recommended concentrations, and test room

The tests were performed in an enclosed room, which was exclusively used and prepared for this test, of approximately 32 m^3.

The product tested was an OPP 15% w/w smoke generator disinfectant. This product is commercialized in Brazil and authorized to sanitize food industry environments without the presence of food or raw materials. The product is presented in a cylindrical tin packaging format. Firing of the foot fumigation chamber was done by removing the seal and wick detachment. Then, it was lit and the smoke immediately dispersed, which quickly covered all the space of the test room. The sanitizer can was lit on the floor according to instructions in the manufacturer's manual.

The manufacturer indicates an OPP concentration of 1 g of product per m^3 of for this type of utility and exposure time of 7 h (the place of application must remain closed for 7 h and, once opened, the environment must be ventilated for at least 15 min before personal entrance). It is also recommended to clean the surfaces and equipment before reusing them.

2.2. Microorganisms used and standardization of the initial inoculum

Ten strains (2 standard strains for the sanitizing tests: *Aspergillus brasiliensis* (ATCC 16404) and *Candida albicans* (ATCC 10231); and 8 strains commonly related to ambient air and food product spoilage: *Aspergillus flavus* (ATCC 9643); *Aspergillus chevalieri* (Syn. *Eurotium chevalieri)* (IMI 211382); *Cladosporium cladosporioides* (IMI 158517); *Lichtheima corymbifera* (Syn. *Absidia corymbifera)* (CCT 4485); *Mucor hiemalis* (CCT 4561); *Penicillium commune* (CCT 7683); *Penicillium polonicum* (NGT 33/12); *Penicillium roqueforti* (IMI 217568)) were used. For preservation and quality assurance of viable cells, all strains were lyophilized and kept under refrigeration during the experiment.

For the initial spore solution preparation, tubes containing Malt Extract Agar (MEA) [glucose, 20 g (Neon, São Paulo, Brazil); peptone, 1 g (Himedia, Mumbai, India); malt extract, 30 g (Bacto™, MD, USA); metal traces, 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 sterile aqueous solution of polysorbate 80% (0.05%). Spore concentration was standardized in 10^2, 10^3, 10^4, and 10^5 spores/mL to evaluate the neutralization efficiency of the sanitizing effect of the product and 10^8 spores/mL for *in vitro* efficacy of the antifungal activity of the smoke generator sanitizer. Neutralization efficiency was confirmed after dilution in 0.1% peptone water [peptone, 0.1 g (Himedia, Mumbai, India); distilled water, 1 L] a Neubauer chamber and confirmed by inoculation in plates containing MEA and incubation for 5 days at 25 °C.

2.3. Evaluation of in vitro antifungal efficacy of the OPP smoke generator

The tests were carried out following the norms of the French protocol NF-T-72281 (Norme Française, NF T 72-281, 2014), with adaptations specific for sanitizers diffused by air. The test is illustrated in Fig. 1.

As carriers of microorganisms, 304 stainless steel discs of 2 cm diameter (TSM inox®, Santa Maria, Brazil), which were previously treated with 5% Triton ™ X-100 (Sigma-Aldrich, USA) for 60 min to remove impurities, were used. The disks were rinsed and left immersed in 70% (v/v) isopropanol for 24 h (Neon, São Paulo, Brazil).

2.3.1. Neutralizer efficacy evaluation

A neutralization step was carried out to ensure that the action of the fumigant agent only occurred during the test exposure time. Neutralization was performed with a solution containing casein tryptone 1 g/L, sodium chloride 8.5 g/L, polysorbate 80% 5 g/L, and a denominated recovery liquid (Jaenisch et al., 2010; Norme Française, NF T 72-281, 2014).

Previous tests were performed to evaluate the neutralization efficiency of the sanitizing effect of the product, as indicated in NF-T-72281. Two discs not contaminated with the fungal inoculum were used for this test. The discs were exposed to the fumigant for 7 h in order for the sanitizer to impregnate them.

Then, the impregnated discs were immersed in 100 mL of the previously described neutralizing liquid (recovery liquid) for 5 min. A 1 mL aliquot of the homogenate was then added to the sterile Petri dishes, followed by 1 mL of the inoculum previously adjusted at 10^2, 10^3, 10^4, and 10^5 spores/mL. Finally, 20 mL of Malt Extract Agar (malt extract, food grade, 30 g/L, 15 g/L agar) was added, homogenized, and left to solidify (pour plating).

The plates were incubated at 25 °C for 5 days. Afterwards, the colonies were counted and the neutralization effectiveness of the results compared with the positive control while following the tests described below.

2.3.2. Evaluation of in vitro antifungal efficacy

The smoke generator sanitizer efficacy test was performed by inoculating discs with 50 µL of a suspension of fungal spores previously adjusted to 10^8 spores/mL followed by the addition of 0.05% reconstituted skim milk powder (Elegê, São Paulo, Brazil), which simulated the presence of organic matter present in the environment. Five discs were inoculated with the fungi to be tested in each experiment. Three discs were exposed to the agent and used to test the sanitizer action. The other two discs were not exposed to the sanitizer and used as positive control of fungal inoculum.

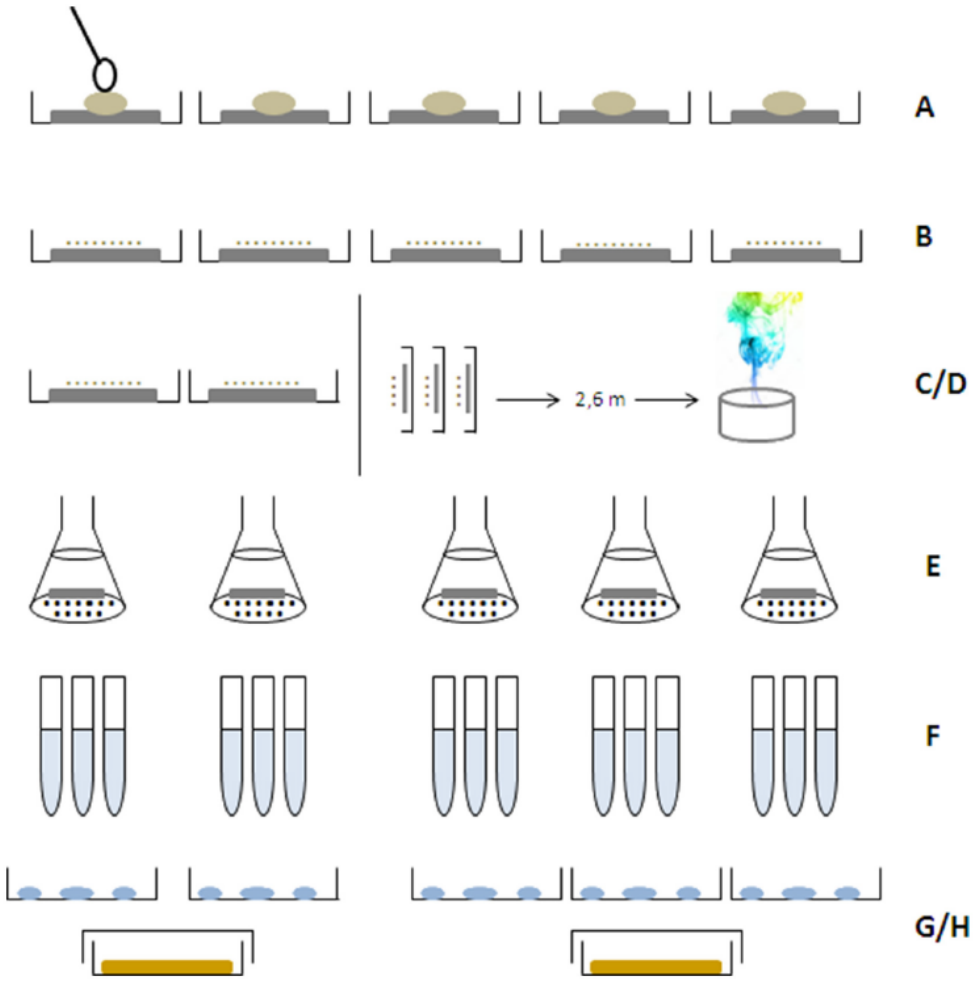

Fig. 1. Scheme of the performance of the test for *in vitro* efficacy tests of air dispersion sanitizers against fungal species according to the standard established by NF-T-72281.
A: Carriers inoculated with 50 µL of spore solution adjusted to 108 spores/mL + addition of 50 µL of reconstituted powdered milk 0.05%. B: Drying at 35 °C for approximately 40 min for adherence of the inoculum in the carriers. C/D: Positive control, without exposure, and test itself, exposed to the sanitizing fumigant. E: Stabilization of microorganisms in neutralizing solution and stirring for 1 minute for detachment and recovery of cells from disc with the aid of glass beads. F: Performed serial dilutions (10-1; 10-2; 10-3). G/H: Inoculation of 1 mL of each dilution in Petri dishes followed by addition of culture medium (pour plating), homogenization and incubation at 25 °C.

Once inoculated, the discs from both tests were placed in the oven at 35 °C for approximately 40 min in order to fix the inoculum. After this period, the discs of the positive control were maintained in the laboratory during the same period that the other discs were exposed to the disinfectant.

Exposure to the fumigating agent was done by placing the discs 2.6 m away from the point where the disinfectant was released. It was vertically positioned with the surface containing the inoculum facing away from the fumigation site.

At the end of the treatment, both inoculated carriers (unexposed and exposed to the fumigant agent) had the viable microorganisms recovered in 100 mL of recovery liquid and stirred for 1 min with 10 g of glass beads. Serial dilutions (10^{-1}; 10^{-2}; 10^{-3}) were performed in 0.1% (m/v) peptone water.

A 1 mL aliquot of each dilution was added to the sterile Petri dishes. Then, 20 mL of Malt Extract Agar (malt extract, food grade, 30 g/L, 15 g/L agar) was added, homogenized, and left to solidify (pour plating).

The plates were incubated at 25 °C for 5 days, the colonies counted, and the results expressed in logarithmic units (log). All tests were performed twice and on different days.

2.3.3. Data analyses

The 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 NF-T-72281, in order to be considered an effective sanitizer, the fumigating agent must be able to reduce, in the tested fungi population (exposed to fumigation), 4 log from the initial amount of microorganisms recovered in the positive control.

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

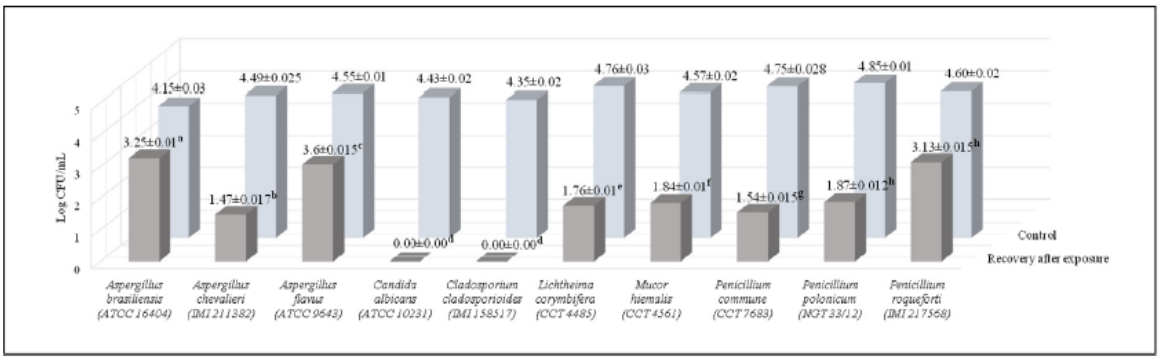

Fig. 2. Average of fungal reduction before and after exposure of different species to a fumigant disinfectant based in orthophenylphenol 15%.
[1]Different lowercase letters in the same line indicate differences observed in the reduction of population among fungi exposed to a same concentration of sanitizer according to Scott-Knott test ($p < 0.05$).

3. Results and discussion

3.1. Evaluation of the in vitro efficacy of the antifungal activity of the fumigant sanitizers

The average fungal reduction and standard deviation obtained after exposure of the microorganisms to the fumigant sanitizer tested is shown in Fig. 2.

The results of the neutralizing efficacy tests were considered satisfactory, since the count of colonies from the standard inoculum was similar to the counts obtained after the neutralization effectiveness test in the presence of the sanitizer (data not shown).

Recovery of viable fungal cells from the positive control after dilution in the recovery liquid was 10^4 CFU/mL of the solution for each fungus tested, thus, to meet the efficiency requirements, no microorganisms should be detected after fumigation. The fumigant sanitizer tested was effective (> 4log reduction) for the two strains, *C. albicans* (ATCC 10231) and *C. cladosporioides* (IMI 158517).

The sensitiveness of these fungal species to other sanitizers has already been reported. Reynolds et al. (2004) achieved a reduction of > 5-log cycles of *Cladosporium* sp. in 5 min of exposure to sodium hypochlorite at 2.4%. Bernardi et al. (2018) obtained a 5-log reduction of *C. cladosporioides* (IMI 158517) and *C. albicans* (ATCC 10231) at 15 min of exposure when testing different sanitizers and concentrations (peracetic acid, benzalkonium chloride, sodium hypochlorite, and quaternary ammonium).

C. albicans is yeast of public health concern related to the spoilage of fruit juices (Paula et al., 2011). *C. cladosporioides* is a psychrophilic fungus commonly related to the deterioration of refrigerated cheese and described as one of the main causes of the so-called "black spot" defect in cured meat (Alía et al., 2016). This fungal genus has been frequently isolated in a variety of meat processing plants and is always found in the air or on equipment surface, although not in raw materials (Sørensen et al., 2008), which demonstrates the relevance of air and environment sanitization.

The sanitizer was not effective against the three *Penicillium* species tested. *P. roqueforti* (IMI 217568) was the most resistant.

Other studies reported resistance of *P. roqueforti* to sanitizers. Bungaard-Nielsen and Nielsen (1995), while using another methodology and liquid sanitizer, showed that ammonium quaternary compounds were inefficient for the *P. roqueforti* strain (IET 11,524). Bernardi et al. (2018) also showed that the strain of *Penicillium roqueforti* (IMI 217568) was resistant to the biguanide sanitizer. On the other hand, these authors reported the efficiency of peracetic acid in controlling this species. Korukluoglu et al. (2006) found similar results.

P. polonicum and *P. commune* are mainly related to the deterioration of cheese (Kure and Skaar, 2000; Kure et al., 2001, 2004; Hayaloglu and Kirbag, 2007). In addition, *P. commune* is associated with the "phenolic defect" that occurs during the maturation of Italian hams (Spotti et al., 1988), in addition to being related to the deterioration of salamis and other matured meat products (Asefa et al., 2009, 2010; Sørensen et al., 2008). Additionally, *P. polonicum* (NGT 33/12) is related to the deterioration of frozen chicken nuggets because it can grow at low temperatures (Saccomori et al., 2015).

The fumigant was capable of a 3-log reduction regarding the positive control for the species of *P. commune* (CCT 7683) and *P. polonicum* (NGT 33/12) used in the test, thus, not achieving the necessary reduction to be considered effective. By comparing the results here with other studies that have evaluated the antifungal activity of chemical sanitizers in liquid form, it is possible to note the low efficacy of the fumigant product used here. Bernardi et al. (2018) obtained a 5-log reduction of these strains at concentrations of 0.5 and 1% of sodium hypochlorite and 1.5 and 3% of peracetic acid tested. However, the authors also observed the resistance of *P. commune* and *P. polonicum* to the biguanide sanitizer tested at concentrations of 2, 3.5, and 5%, where only the 1-log reduction of the strains. No studies regarding phenol-based compounds were found for comparison.

At high concentrations, phenolic compounds act as macroscopic protoplasmic venom, penetrating and rupturing the cell wall of microorganisms and precipitating cellular proteins. Low concentrations of phenol and phenol derivatives of higher molecular weight cause bacterial death by inactivating essential enzyme systems and extravagating essential cell wall metabolites. These compounds are bacteriostatic at lower concentrations, and bactericidal and fungicidal at higher concentrations (Paulino, 2006; Kuana, 2009). However, data on fungal sensitivity to OPP and its mechanism of action are still limited.

The zygomycetes *M. hiemalis* (CCT 4561) and L. *corymbifera* (CCT 4485), which are considered indicators of hygienic ambient air quality, and *A. chevalieri* (IMI 211382), a potentially deteriorating species of generally low water activity aw and bakery products (Jahn et al., 2013; Garcia et al., 2018), showed a 3-log reduction in relation to the positive control. In other protocols for testing sanitizers, such as liquid sanitizers that follow the European Committee for Standardization (CEN) model, the 3-log reduction in relation to the initial control count would be enough to be considered an effective sanitizer (European Standard, n. 13697, 2001). However, this level of reduction is not sufficient according to the French Protocol NF T 72-281 (Norme Française, NF T 72-281, 2014), which is the only regulation available for testing aerial dispersion sanitizers, and that request 4-log reduction.

Aspergillus flavus (ATCC 9643) and *Aspergillus brasiliensis* (ATCC 16404) were also resistant to the product, with a reduction of only 1 log. This can be extensively compared with other studies available in the literature, which used different methodologies but also tested fumigants. Pornpukdeewattana et al. (2017) tested the volatile components of rice vinegar as a fumigant for reducing *A. flavus* in maize grains and achieved a total reduction of the initial conidial population (6 log

CFU/mL) of this species after 5 h of exposure. Similar results were found by Boukaew et al. (2017), who obtained an inhibition of this fungus after 6 h of fumigation with essential oils (clove and vatica).

A. flavus is one of most widespread and important fungi related to food spoilage and aflatoxin production in cereals in the world (Pitt and Hocking, 2009). *A. brasiliensis* (previously classified as *Aspergillus niger*) is also related to the post-harvest deterioration of fresh fruits, such as pears, apples, peaches, grapes, (Snowdon, 1990) tomatoes (Muhammad et al., 2004), and as a cause of deterioration in soft cheeses (Hocking and Faedo, 1992).

4. Conclusion

This study aimed to verify the efficacy of an orthophenylphenol-based smoke generator sanitizer against fungal species commonly present in the air of food industries. The product was effective against *C. albicans* and *C. cladosporioides*, although it was unable to reduce 4 log of the other species tested. Despite the feasibility and easy diffusion of smoke generator sanitizers in food production facilities, more attention should be paid to fungal control when evaluating their efficacy. This study showed variable sensitivity of food spoilage fungal species to the sanitizer tested. Therefore, we emphasize the importance of previously testing the target microorganism (causing a specific problem in the food industry) with the sanitizer aimed at controlling it.

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Conflicts of interest

The authors declare no conflict of interest.

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5.3 ARTIGO 3 – ANTIFUNGAL ACTIVITY OF COMMERCIAL SANITIZERS AGAINST STRAINS OF *PENICILLIUM ROQUEFORTI*, *PENICILLIUM PANEUM*, *HYPHOPICHIA BURTONII*, AND *ASPERGILLUS PSEUDOGLAUCUS*: BAKERY SPOILAGE FUNGI

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

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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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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

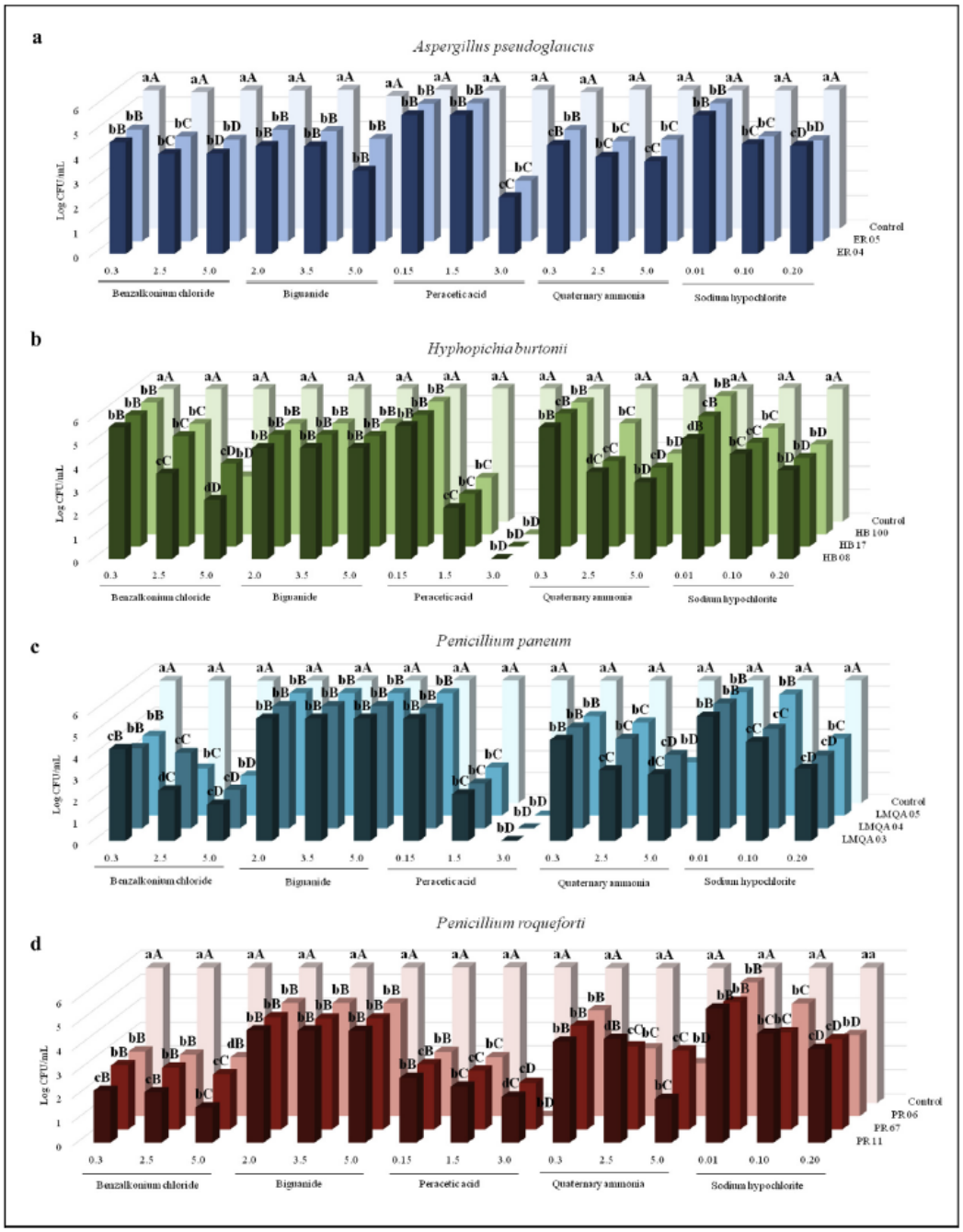

Fig. 1. Efficacy of commercial sanitizers against bakery spoilage fungi: (a) *Aspergillus pseudoglaucus* (ER 04; ER 05); (b) *Hyphopichia burtonii* (HB 08; HB 17; HB 100); (c) *Penicillium paneum* (LMQA 03; LMQA 04; LMQA 05); and (d) *Penicillium roqueforti* (PR 06; PR 11; PR 67) tested with the recommended 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.

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5.4 ARTIGO 4 – CONTROL OF CHEESE AND MEAT PRODUCT SPOILAGE FUNGI BY SANITIZERS

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Control of cheese and meat product spoilage fungi by sanitizers

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Research highlights:

- Commercial sanitizers were tested against cheese and cured meat spoilage fungi.
- The antifungal efficacy was variable according to type and sanitizers concentration tested.
- Variation in the susceptibility was observed among strains of a same species.
- Peracetic acid was the most effective sanitizer.

Abstract

The use of adequate sanitizers would be an alternative to prevent the early spoilage and economic losses of dairy and meat products industries; however, specific information on the sensitivity of fungi commonly related to the spoilage of products of animal origin to sanitizing agents is scarce. This work evaluated the antifungal capacity of different sanitizers allowed in the food industry, against species involved in the spoilage of these products. Tests were carried out according to the protocol for testing antifungal effects of chemical sanitizers of the European Committee for Standardization (CEN) and the French Standard NF-T-72281. *Aspergillus* and *Penicillium* species commonly related to the spoilage of dairy and meat products

were tested against benzalkonium chloride, biguanide, peracetic acid, quaternary ammonium, sodium hypochlorite, and a smoke generator agent based on orthophenylphenol. Peracetic acid was the most effective sanitizer considering the liquid sanitizers, species, and concentrations evaluated for dairy product spoilage fungi. The spoilage fungi isolated from meat products were resistant to most of the sanitizers and concentrations tested. Biguanide, sodium hypochlorite and the fumigant showed very low efficacy and should not be the compounds of choice for controlling spoilage fungi of cheese and cured meat product.

Keywords: sanitizers; fungal control; spoilage; dairy products; meat products.

1. Introduction

Within the context of deterioration caused by fungi in dairy and meat products, the genera *Aspergillus, Candida*, *Cladosporium*, *Mucor*, *Penicillium, Geotrichum*, and *Trichoderma* are commonly mentioned (Deák, 2008; Pitt & Hocking, 2009; Jahn, Garcia & Copetti, 2017; Papagianni et al., 2007; Sørensen et al., 2008; Perrone et al., 2015; Vila, Pose, Segura, & Ludemann, 2016; Vipotnik & Rodríguez and Rodrigues, 2017; Parussolo, Bernardi, Garcia, Stefanello, Silva, & Copetti, 2019a). The fungal genera involved in the spoilage of these products originate mainly from the production environment, which includes air, work surfaces, equipment, personnel, raw materials, and ingredients (Kure et al., 2004; Vacheyrou et al., 2011; Jahn et al., 2017 Battilani et al., 2007; Sorensen et al., 2008).

The typical pH range (4.3 - 4.9) and high moisture content of cheese (Jacobsen & Norvhus, 1996), and tolerance to low pH and high salt concentration in cured meat products (Pitt & Hocking, 1999), favors the development of filamentous fungi. Industries seek to control these microorganisms by adding antimicrobial compounds and preservatives during product manufacturing (Costa et al., 2017).

However, consumer demands for preservative-free products in recent years have increased, as healthier and safer food requirements have stipulated more natural ingredients in processed foods (Resa, Jagusb, & Gerschenson, 2014). As a result, the hygiene of environment air and work surfaces have gained prominence. By adopting hygienic-sanitary measures, such as employing effective cleaning methods and sanitization processes, it is possible to reduce the

contamination of surfaces and environments to a safe level, which will impact the microbial load of food and influence its shelf life.

However, data on the efficacy of commercial sanitizers against fungal species associated to the spoilage of fresh and cured products of animal origin are very limited. On the opposite, knowledge on the fungal sensitivity for each class of sanitizers with permitted application in the food industry is extremely useful in guiding hygiene processes and improving the sanitary status of industries.

Thus, the aim of this study was to evaluate the antifungal activity of different classes of commercial sanitizers (liquid and fumigant), both with permitted use in food industries, on relevant spoilage fungi of products of animal origin. Additionally, the existence of variation in the inter and intraspecific resistance to the sanitizers and concentrations employed was verified.

2. Materials and method

2.1 Microorganisms, liquids and fumigant sanitizers, and neutralizing solutions

The fungi used in this study are listed in Table 1. The preparation and standardization of the inoculum was performed according to Bernardi et al. (2018; 2019b).

Five different chemical sanitizers (Table 2) were chosen for convenience among the available in the Brazilian market and with authorized use in the food industry by the National Health Surveillance Agency (ANVISA) and are commonly allowed by other international agencies, such as the European Union and the United States (CDC, 2008; Jeffrey, 1995 White, 1999). These agents were tested in the minimum, intermediate (mean) and maximum values indicated on the label. Additionally, efficacy of higher concentrations of sodium hypochlorite [(0.5%; 5000 ppm), 1.0%; 10000 ppm)] was also checked.

For the fumigant sanitizer, an orthophenylphenol (OPP)-based smoke generator (15% w/w) the concentration established by the manufacturer was used (1g/m^3) and the time of exposition (7 h) was the maximum indicated on label. The tests were performed in an enclosed room (~32 m^3) (Bernardi et al., 2019a). This

product is authorized in Brazil to food industry environments sanitization, in the absence of food or raw materials.

For each sanitizing principle, neutralizing solutions were used to restrict the agents action to the time of contact specified in the test protocol (Jaenisch et al., 2010; Norme Française, 2014).

2.3 *In vitro* antifungal activity efficacy of liquid sanitizers

Tests were carried out according to the standards established for antimicrobial testing of chemical sanitizers by the European Committee for Standardization (CEN) (European Standard 13697, 2001), following adaptations described in Bernardi et al. (2018). As support for the microorganisms, 304 stainless steel discs 2 cm in diameter (TSM laser®, Santa Maria, Brazil) were used. Results were expressed in logarithmic units (log).

2.4 *In vitro* antifungal efficacy of a fumigant sanitizer

The smoke generator sanitizer efficacy test was performed by inoculating discs with 50 µL of a suspension of fungal spores previously adjusted to 10^7 spores/mL. Five discs were inoculated with the fungi to be tested in each experiment. Three discs were exposed to the agent and used to test the sanitizer action. The other two discs were not exposed to the sanitizer and used as positive control of fungal inoculum.

Exposure to the fumigating agent was done by placing the discs 2.6 m away from the point where the disinfectant was released. It was vertically positioned with the surface containing the inoculum facing away from the fumigation site.

At the end of the treatment, both inoculated carriers (unexposed and exposed to the fumigant agent) had the viable microorganisms recovered in 100 mL of recovery liquid and stirred for 1 min with 10 g of glass beads. Serial dilutions (10^{-1}; 10^{-2}; 10^{-3}) were performed in 0.1% (m/v) peptone water.

A 1 mL aliquot of each dilution was added to the sterile Petri dishes. Then, 20 ml of Malt Extract Agar (malt extract, food grade, 30 g/L, 15 g/L agar) was added, homogenized, and left to solidify (pour plating).

The plates were incubated at 25°C for 5 days, the colonies counted, and the results expressed in logarithmic units (log). All tests were performed twice and on different days.

2.5 Data analyses

2.5.1 Statistical analyses

To be effective, a liquid sanitizer should reduce at least 3 log (99.9% reduction) of the initial number of fungi recovered in the positive control (European Standard 13697, 2001). For fumigant agents, the expected reduction should be 4 log (99.99% reduction) from the initial number of fungi recovered in the positive control (NF-T-72281).

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

2.5.2 Antifungal efficacy

The 5-point scale proposed by Bernardi et al. (2018) was adopted to evaluate the antifungal efficacy of sanitizers.

3. Results and discussion

3.1 *In vitro* antifungal efficacy of liquid sanitizers and OPP smoke generator sanitizer

3.1.1 Cheese spoilage fungi

Variations were observed in antifungal activity among the sanitizers used in the test, between the concentrations applied, and susceptibility of the species and isolates from cheese spoilage strains tested (Figure 1).

Strains of *P. roqueforti* and *P. commune*, in general, showed differences in sensitivity to the sanitizers used, which shows a variation in the susceptibility pattern for most of the agents at the concentrations tested.

In relation to the susceptibility of strains isolated from spoiled cheese (PR 02, PR 03, and PC 04) to peracetic acid, higher resistance was observed in *P. roqueforti* compared to *P. commune*, especially at intermediate concentration. Peracetic acid was able to reduce approximately 3 logs for *P. roqueforti* PR 02 and PR 03 at the intermediate concentration (reduced efficacy) and at the highest concentration obtained reductions of about 3 log (good efficacy). For *P. commune* PC 04, this sanitizer obtained reductions of more than 4 log (maximum efficacy) for the intermediate concentration and above.

Similar results were found regarding sodium hypochlorite at the highest concentration tested (1%), although sodium hypochlorite is commonly used in food industries at concentrations ranging from 0.02% to 0.08% for equipment and utensil sanitation and up to 0.12% for facility cleaning (Menegaro et al., 2016). However, this agent was able to reduce more than 4 log in relation to the positive control (maximum efficacy) both for strains of *P. roqueforti* PR 02 and PR 03 and *P. commune* PC 04 at the highest concentration (1%), and one of the control alternatives for fungi that present resistance to lower concentrations and other sanitizers may raise concentrations beyond the recommended values. Nevertheless, we must remember that sodium hypochlorite is naturally highly corrosive and high concentration solutions can shorten the life of treated equipment (Bernardi et al., 2019b).

Considering the *in vitro* results, peracetic acid can be considered suitable sanitizer for decontamination of dairy industry environments when the spoilage fungi to be controlled (or the predominant contaminating mycobiota) are *P. roqueforti* and/or *P. commune*. Both agents may be alternated at regular intervals to avoid the selection pressure of a single agent, which may lead to resistance to the applied sanitizer continuously.

The differences in sensitivity between *P. roqueforti* and *P. commune* strains can also be observed for the quaternary ammonia compound and its derivative, benzalkonium chloride. For *P. commune* PC 04 and NGT 16/12 strains, both quaternary ammonium and benzalkonium chloride were capable of reducing 3 log (good efficacy) at the highest concentrations. However, for the *P. roqueforti* PR 02 and PR 03 strains, which were isolated from the spoiled cheese, benzalkonium

chloride was not effective even at the highest concentration (poor efficacy). Moreover, quaternary ammonium was only able to reduce 3 log (good efficacy) at the highest concentration.

These results differ from a study by Bernardi et al. (2019b), where the efficacy of quaternary ammonia and benzalkonium chloride against *P. roqueforti* strains isolated from spoiled bread was evaluated. The authors reported that these sanitizers met reduction requirements, reducing between 3 and 4 log, respectively, at the intermediate and highest concentrations. Additionally, Korukluoglu et al. (2006) reported that 5 strains of *P. roqueforti* were susceptible to benzalkonium chloride and obtained effective inhibition at a concentration of 2% between 2 and 11 min of exposure, in which the strain from cheese was one of the most sensitive. On the other hand, the unsatisfactory results observed in this study of *P. roqueforti* isolated from cheese is similar to the observations by Bundgaard-Nielsen & Nielsen (1996), who used a different methodology and reported that ammonium quaternary compounds were ineffective against *P. roqueforti* (IET 11524).

Once again, we highlight the importance of knowing the sensitivity of the strains present in the environment or spoiling the products elaborated in each food industry to choose the most suitable sanitizer for each specific situation (Bernardi et al., 2018).

In addition, the highest sensitivity showed by the strains acquired from a culture collection of *P. roqueforti* (IMI 217568) and *P. commune* (CCT 7683) illustrates the variation between the strains causing spoilage problems in industries and those commercially available. Moreover, both sodium hypochlorite and peracetic acids were able to eliminate the population (5 log, maximum efficacy) at intermediate concentrations for peracetic acid and at high concentrations beyond the limit for sodium hypochlorite tested.

Nevertheless, in the commercial strains, benzalkonium chloride and biguanide had reduced efficacy for the *P. commune* (CCT 7683) strain. On the other hand, *P. roqueforti* (IMI 217568) was sensitive to biguanide, unlike the other fungal isolates used in this study (3 log reduction at the highest concentration tested, good efficacy). This reduced biguanide antifungal efficacy was already reported by Bernardi et al. (2019b) with other fungal species. This corroborates that this agent should not be chosen when the goal is fungal control in the food industry, even in the dairy industry.

Synthesizing the results obtained in the *in vitro* tests, *P. commune* causes the most problems in certain dairy products, therefore, the agent of choice to control it is sodium hypochlorite at the at high concentrations beyond the limit (0.5% and 1%), which may be intercalated with peracetic acid at concentrations of 1.5% and 3%. Alternatively, benzalkonium chloride may also be applied at its maximum concentration (5%) (supplementary material).

To control *P. roqueforti*, sodium hypochlorite at the maximum concentration (1%) and alternatively, quaternary ammonium (5%) and peracetic acid (3%) can be used.

The average fungal reduction obtained after exposure of the microorganisms to the fumigant (OPP) is shown in Figure 2.

The fumigant was ineffective against the species tested. There was no significant variation of resistance among the species used except for a difference of 1 log reduction for *P. commune* (poor efficacy) in relation to *P. roqueforti* (inefficacy).

Smoke generator sanitizers are easy to handle, can access hard-to-reach places, and exert their effect during the exposure period while leaving little or no residue. These agents are an important alternative for microbial control in the food industry (Sholberg et al., 2004), although they are not recommended for controlling the most common spoilage fungi of the dairy industry.

As observed throughout this study, the isolation of the fungal strain from the deteriorated product and evaluation of its sensitivity is of extreme importance, since sanitizers vary in their forms of action according to the microorganisms involved. Contrary to what was reported here, Pornpukdeewattana et al. (2017) employed a different methodology but with a fumigant agent based on rice vinegar and other fungal strains. The authors achieved a reduction of 6 log of *Aspergillus flavus* from corn kernels after 5 h of exposure. Similar results were found by Boukaew et al. (2017), who obtained an inhibition of this fungus after 6 h of fumigation with essential oils (clove and vatica). Low antifungal efficiency of an OPP fumigant was also reported by Bernardi et al. (2019a).

3.1.2 Cured meat products spoilage fungi

Corroborating the results obtained with fungal strains from spoiled cheese, variations were observed in antifungal activity among the sanitizers used in the test between the concentrations applied and susceptibility of the species and strains of cured meat products (Figure 3).

In a recent study, Parussolo, Bernardi, Garcia, Stefanello, Silva, & Copetti (2019a) investigated the fungal contamination present on the surface of dry fermented sausages produced in Brazil and found that *A. westerdijkiae* was predominant in salami samples and in the air of the maturation chamber from a studied industry. This factor was considered alarming by the researchers, since this species represents a concern in relation to ochratoxin A (OTA) production.

The air has been described as one of the main factors for promoting spore dispersion, which are deposited and adhere to the surface of meat products during maturation, affecting the quality of final products (Asefa et al., 2010; Battilani et al., 2007; Samson, Frisvad, & Hoekstra, 2004; Sørensen, Jacobsen, Nielsen, Frisvad, & Kock, 2008; Wigmann et al., 2018; Parussolo et al., 2019a). Adequate cleaning and sanitizing can be a way to control this problem (Parussolo et al., 2019a).

Nevertheless, as observed in our study, the OTA-producing strains of *A. westerdijkiae* isolated from the spoiled dry fermented sausages (Parussolo et al., 2019b) were the most resistant to liquid sanitizers commonly used in the hygiene process in the food industry. This was not the case for peracetic acid at concentrations of 1.5 and 3.0% and sodium hypochlorite above the usually recommended values (0.5 and 1%). These agents have shown efficacy against the strain AW 03 by reducing 3 log in relation to the positive control (good efficacy) for peracetic acid, and even reductions above 5 log (maximum efficacy) for the sodium hypochlorite in maximum concentration (1%). For strains AW 01 and AW, no sanitizing agent was capable of reducing more than 3 log (poor efficacy), thus, there was a resistance check between the strains.

The OPP based on the tested fumigant obtained significant reductions of 3 log in relation to the control positive for both strains of *A. westerdijkiae* (AW 01; AW 02; AW 03) (Figure 4). However, this was different from the liquid sanitizers used in the tests with strains isolated from the meat product that did not show good efficacy, which is unlike what occurred for strains isolated from cheese used in this work and in the previous study by Bernardi et al. (2019a). Moreover, although the OPP-based fumigant did not meet the requirement of 4 log to be considered an antimicrobial

fumigant agent by NF-T-72281, as it achieved the limit set by CEN for liquids sanitizers. Therefore, it could be a good agent to be used concomitantly with other sanitizing agents, such as peracetic acids at the maximum concentration (3%), resulting in good sanitation of the production and maturation environment of meat products. This result is similar to what was reported by Parussolo, Bernardi, Garcia, Stefanello, Silva, & Copetti, (2019a), who reported that one of the industries evaluated achieved good reduction in the presence of *A. westerdijkiae* using sodium hypochlorite and an OPP-based fumigant.

We emphasize once again that maintaining good air quality in the chambers is the most effective way of reducing the presence of undesirable fungal spores in the production and maturation environment. However, as the use of OPP in the presence of food products is not yet authorized due to the lack of safety studies (Bernardi et al., 2019a), its use would be possible at the end of each maturation batch of the cured meat product, which is when the maturation room is empty, thus also possibly improving the efficacy of the hygiene process. After its time of action, another sanitizer can be dispersed, subsequently rinsing and allowing use of the environment again.

In relation to the spoilage fungi and toxigenic potential of the genus *Penicillium* tested: *P. polonicum* (PP 02) isolated from spoiled ham and *P. polonicum* (NGT 23/12) isolated from spoiled chicken nuggets and with known toxigenic potential, which produce verrucosidin in addition to other extrolites (Wigmann, Saccomori, Bernardi, Frisvad, & Copetti, 2015), were very resistant to all tested products, both the liquid and smoke generators (inefficacy). Ingredients added during the preparation of chicken nuggets may be a source of contamination since *P. polonicum* is a species frequently isolated from maize and wheat from tropical countries (Samson & Frisvad, 2004). These cereals are present in the breading flour of nuggets (Barbut, 2002), which can spread the fungal spores in the factory environment, including the air.

Due to resistance of the species of *P. polonicum* to commonly used and authorized concentrations, one of the possible solutions to control these fungi in the environment is the use of concentrations above the usually recommended, as in the case of the increased concentrations of sodium hypochlorite. At the highest concentrations of 0.5 and 1%, this sanitizer was effective in controlling the tested strains. At a concentration of 1%, it was capable of reducing more than 3 log of

strains of *P. polonicum* PP 02 and NGT 23/12 (good efficacy). Nevertheless, we must remember that chlorine can be corrosive to surfaces and irritate the skin (Bernardi et al., 2019b).

In addition to the genus *Penicillium*, the xerophilic species of *Aspergillus*, especially those with the *Eurotium* sexual form, are also worth mentioning in meat spoilage. Xerophilic fungi, such as *A. pseudoglaucus* (formerly *E. repens*), are commonly related to the spoilage of low water activity products. Parussolo, Bernardi, Garcia, Stefanello, Silva, & Copetti, (2019a) found 40% of *A. pseudoglaucus* strains among the total external air isolates in the salami industry and this was present until the 7th day of maturation in the chambers.

In addition to the *A. westerdijkiae* isolated strains, there was a variation of resistance among the tested strains of *A. pseudoglaucus*, which also proved to be a resistant strain, corroborating with Bernardi, Stefanello, Lemos, Garcia, & Copetti (2019b), where strains of this fungus isolated from bakery product were the most resistant to sanitizers.

The ER 01 strain was shown to be more resistant in comparison to the ER 03 strain for all the liquid sanitizers tested. On the other hand, the ER 01 strain resisted to most sanitizing products with the exception of the peracetic acid at the maximum concentration of 3% (good efficacy). The ER 03 strain presented low resistance to the sanitizers at the highest concentrations tested, with reductions close to 3 log (reduced efficacy) for all products, with the exception of biguanide and the fumigant agent. Therefore, for cases where the fungus problem is *A. pseudoglaucus*, the most indicated sanitizer is the peracetic acid at the maximum concentration (3%), and it can be alternated with benzalkonium chloride and quaternary ammonia in their maximum concentrations of 5% (supplementary material).

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4. Conclusion

This study evaluated the antifungal activity of different classes of liquid sanitizers and a smoke generator sanitizer, both with permitted use in the food industry against the main fungi involved in the spoilage of dairy and cured products. Peracetic acid was the most effective sanitizer in the conditions evaluated in this work. In the case of liquid sanitizers, variations were observed among isolates of a species and, therefore, it is recommended that, in the presence of recurrent problems

of fungal spoilage in dairy and cured meat products, these fungi should be isolated from the moldy cheese and cured meat and their sensitivity to commercial sanitizers tested.

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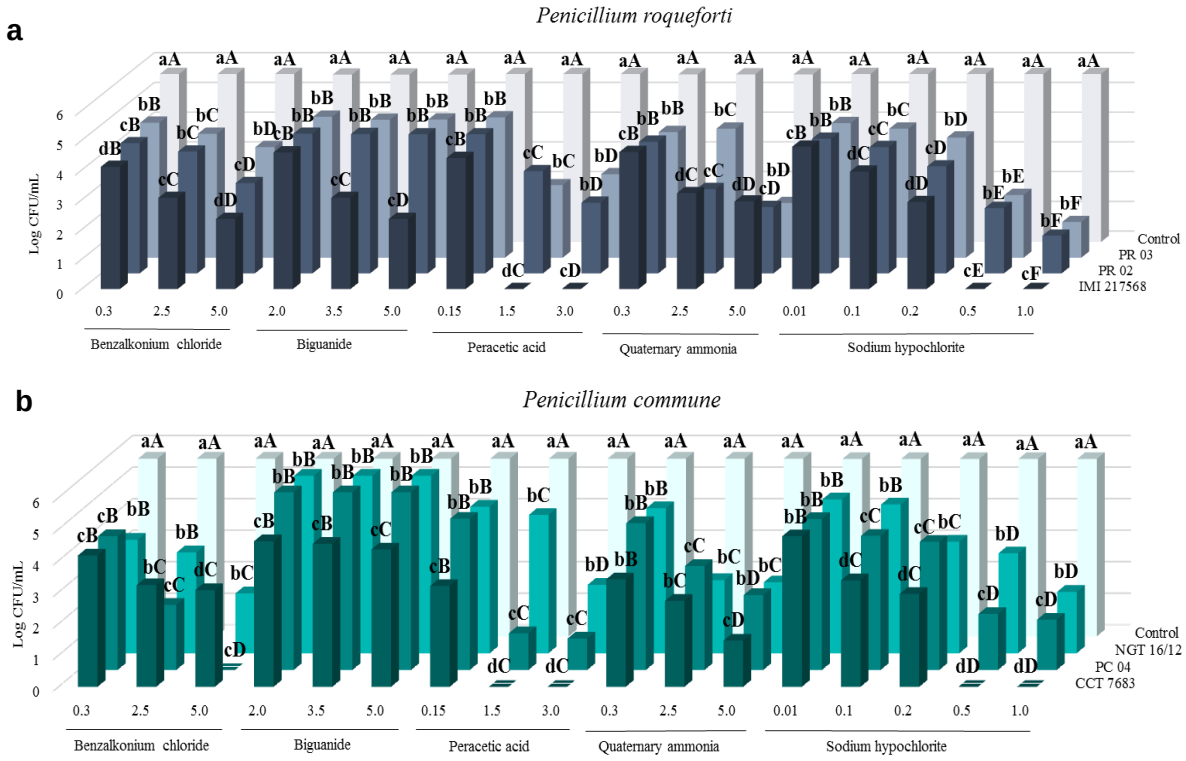
Table 1. Strains used in the commercial sanitizers efficacy test and source of isolation.

Fungi	Strain	Source of isolation
Penicillium commune	PC 04	Spoiled mozzarela cheese, Brazil
Penicillium commune	NGT 16/12	Spoiled chicken nugget, Brazil
Penicillium commune	CCT 7683	Culture bank, Brazil
Penicillium roqueforti	PR 02	Spoiled mozzarela cheese, Brazil
Penicillium roqueforti	PR 03	Spoiled mozzarela cheese, Brazil
Penicillium roqueforti	IMI 217568	Stilton cheese, Brazil
Aspergillus westerdijkiae	AW 01	Spoiled dry fermented sausage, Brazil
Aspergillus westerdijkiae	AW 02	Spoiled dry fermented sausage, Brazil
Aspergillus westerdijkiae	AW 03	Spoiled dry fermented sausage, Brazil
Penicillium polonicum	PP 02	Spoiled ham, Brazil
Penicillium polonicum	NGT 23/12	Spoiled chicken nugget, Brazil
Aspergillus pseudoglaucus	ER 01	Spoiled meat product, Brazil
Aspergillus pseudoglaucus	ER 03	Spoiled meat product, Brazil

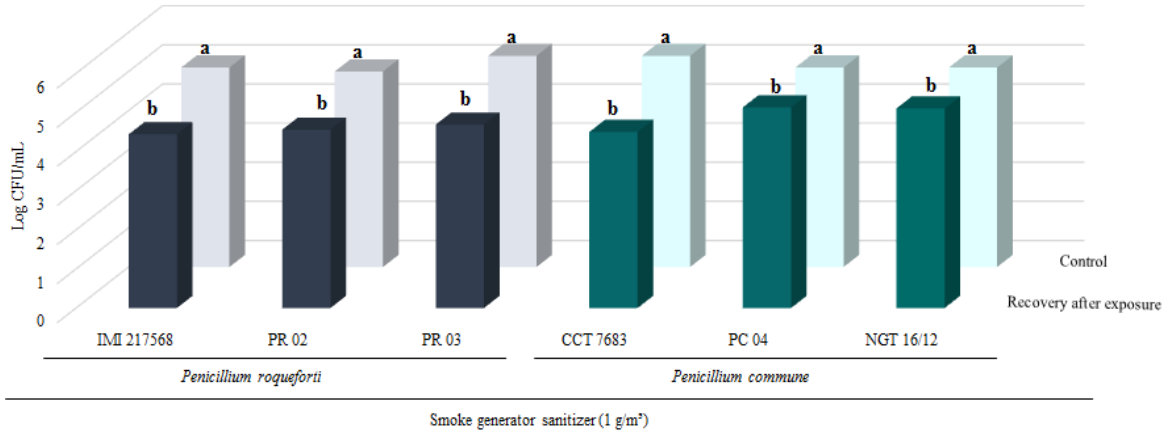
Table 2. Sanitizers with the permitted use in food production environments and concentrations tested.

Sanitizer	Active principle	Concentrations
Benzalkonium chloride	Benzalkonium chloride	0.3%, 2.5%, 5%
Biguanide	Hexamethylene biguanide hydrochloride	0.3%, 2.5%, 5%
Peracetic acid	Peracetic acid, hidrogen peroxide, Acetic acid	0.15%, 1.5%, 3%
Quaternary ammonia	Quaternary ammonia, tensoative and water.	0.3%, 2.5%, 5%
Sodium hypochlorite	Sodium hypochlorite 10 to 12% of active chlorine	0.01%, 0.1%, 0.2%



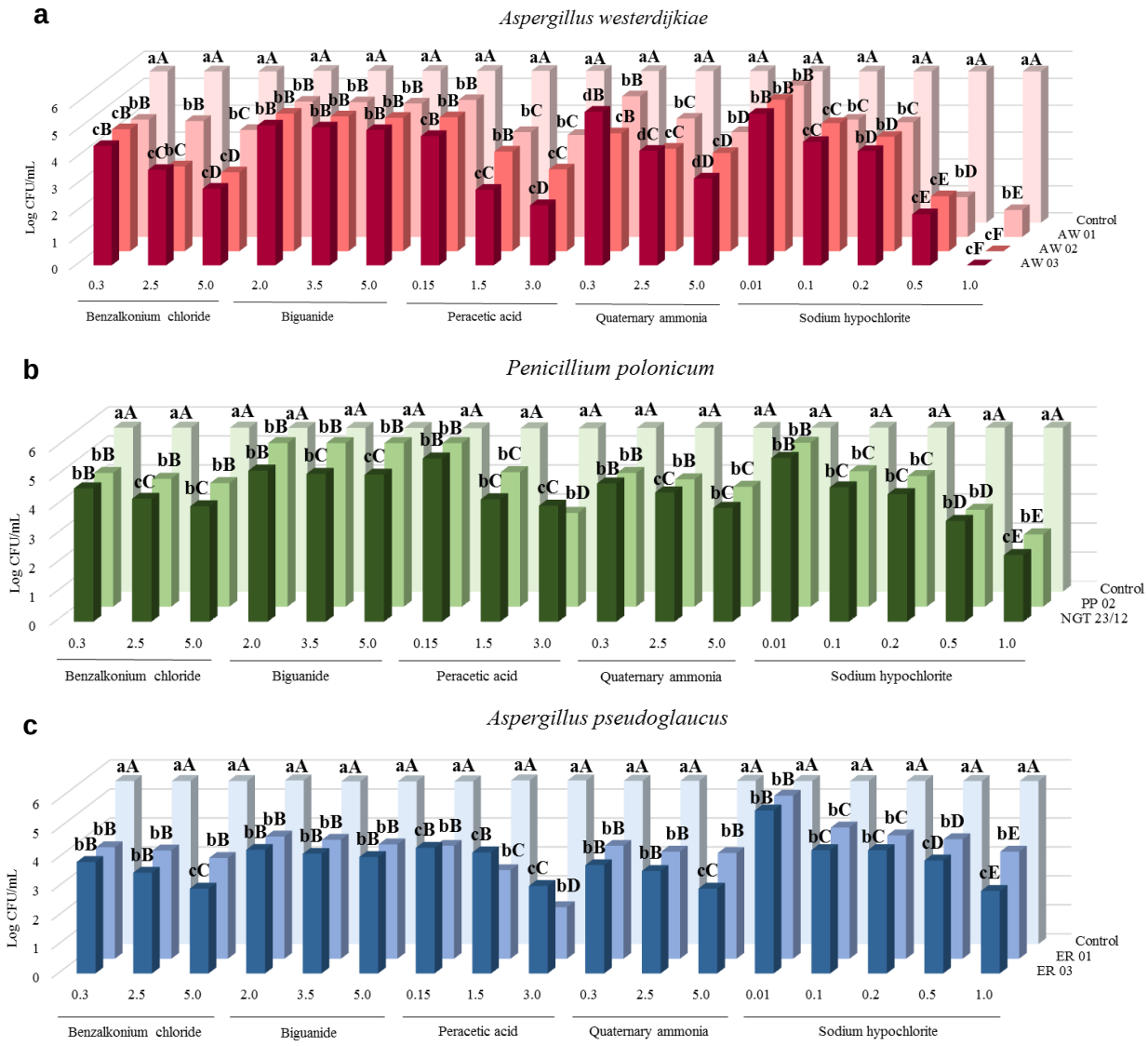
[1]Different lowercase letters in the same column indicate differences between the tested isolates of the same concentration of sanitizers employed according to Scott-Knott test ($p<0.05$).
[2]Different uppercase letters in the same line indicate differences between the concentrations of sanitizers tested for the same isolates according to Scott-Knott test ($p<0.05$)

Figure 1. Efficacy of liquid sanitizers against dairy spoilage fungi: (a) *P. roqueforti* (IMI 217568; PR 02; PR 03) and (b) *P. commune* (CCT 7683; PC 04; NGT 16/12); tested with the recommended use concentrations for benzalkonium chloride; biguanide; peracetic acid; quaternary ammonium; sodium hypochlorite.



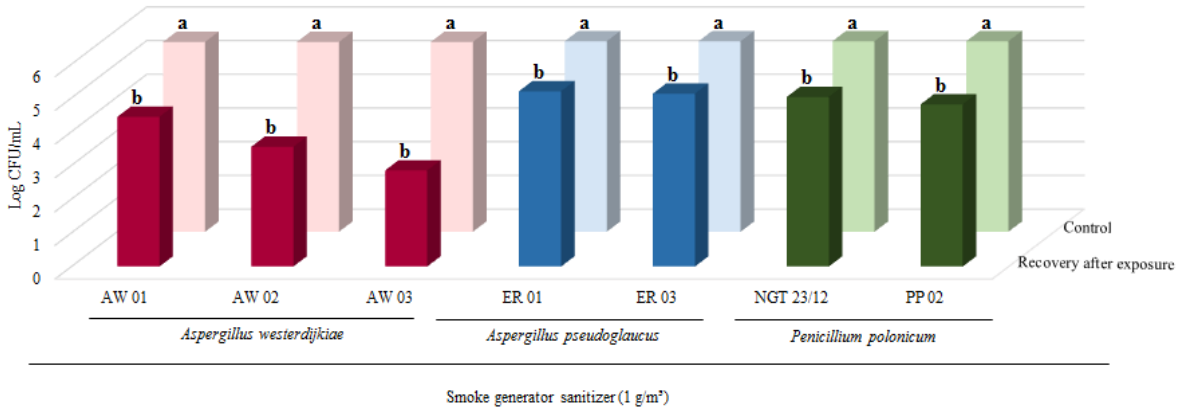
[1]Different lowercase letters in the same column indicate differences between the tested isolates of the same concentration of sanitizers employed according to Scott-Knott test ($p<0.05$).

Figure 2. Efficacy of the smoke generator sanitizer against dairy spoilage fungi: *P. roqueforti* (IMI 217568; PR 02; PR 03) and *P. commune* (CCT 7683; PC 04; NGT 16/12); tested with the recommended use concentration for the OPP fumigant.



[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 ($p<0.05$).

Figure 3. Efficacy of liquid sanitizers against cured meat spoilage fungi: (a) *A. westerdijkiae* (AW 01; AW 02; AW 03), (b) *P. polonicum* (PP 02; NGT 23/12), and (c) *A. pseudoglaucus* (ER 01; ER 03); tested with the recommended use concentrations for 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 ($p<0.05$).

Figure 4. Efficacy of the smoke generator sanitizer against cured meat spoilage fungi: *A. westerdijkiae* (AW 01; AW 02; AW 03), *P. polonicum* (PP 02; NGT 23/12), and *A. pseudoglaucus* (ER 01; ER 03); tested with the recommended use concentration for the OPP fumigant.

5.5 ARTIGO 5 – FOOD INDUSTRY SPOILAGE FUNGI CONTROL THROUGH FACILITY SANITIZATION

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Food industry spoilage fungi control through facility sanitization

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Research highlights:

- Airborne fungi play a crucial role in the food contamination during the processing.
- The choice of antifungal agents is relevant for the control of fungal spoilage.
- The antifungal efficacy varies according to type and sanitizers concentration.
- Peracetic acid is the most effective antifungal sanitizer allowed in food industries.

Abstract

The choice of sanitizing agents with adequate antifungal activity is a relevant step for the control of losses related to fungal spoilage in the food industry. Spoilage fungi usually gain access to the premises through contaminated raw materials. These organisms disperse easily through the air and can deposit themselves onto the surface of freshly made products. If they find the proper conditions for growth, they will form colonies that release more fungal propagules into the environment. Therefore, adequate hygiene of the industrial environment is of extreme relevance to prevent and control fungal deterioration in the food industry. This work presents recent information on the antifungal activity of different classes of commercial sanitizers with permitted use in the food industry against the main fungi involved in spoiling bakery, dairy, and meat products.

Keywords: sanitizers; fungal control; spoilage; bakery product; dairy; meat product.

Introduction

The fungal species responsible for the spoilage of industrialized foods tends to vary according to the particularities of the food product (e.g. composition, physicochemical characteristics, and processing to which it was subjected to) [1]. The problem begins with the contamination of the food, and the subsequent multiplication of these undesirable microorganisms can lead to product deterioration, causing considerable economic losses, in addition to being a danger to consumer health when mycotoxin-producing fungi are involved [2,3,4].

The environment of food production and processing is usually one of the main sources of contamination, being the air an important disseminator of fungal spores [4,5,6,7,8,9]. These fungal propagules commonly enter industry environments mostly through contaminated raw materials and are released in the form of aerosols in the early stages of production and tend to settle on the surface of products over time.

In this sense, non-effective hygienic control of the factory environment and lack of care in selecting raw materials favor the development of fungal microbiota with potential deterioration [10]. Detecting potential spoilers and adopting effective measures for reducing microbial contamination in production environments is one of the most effective ways to minimize the spoilage of food products, thus increasing product shelf life. Choosing the most effective antifungal sanitizers and defining adequate concentrations of use are essential for successful fungal control in the food industry, which directly reflects economic losses. Even then, successful disinfection still depends not only on the characteristics of the sanitizer and the way it is employed, but on the characteristics of the microorganisms present on the site. Knowledge of the fungal sensitivity for each class of sanitizing agent appropriate for use in the food industry is extremely useful for guiding hygiene processes, thus improving sanitation efficacy [11].

Authorized sanitizers used in the food industry

One of the basic concerns related to food production is regarding the use of sanitizers that are harmless to consumers, and the guidelines are defined in specific hygiene regulations. Every substance and chemical should be assessed in the specific context of food production. Because some chemical cleaners and sanitizers have residual toxicity, specific care must be taken during the sanitization process to minimize these hazards, how to use personal protective equipment, perform a careful rinse and wait long enough for volatile sanitizers to evaporate before resuming subsequent production. As some traces are technically unavoidable, it is not appropriate to attempt any steps to achieve zero traces in food [12].

Another aspect to consider is the stability and damage that a sanitizing agent can cause on the surface of equipment since this injury may later propitiate the accumulation of organic matter and favor the insertion of microbial biofilm. For example, sodium hypochlorite, which is regarded as a broad-spectrum and low-cost sanitizer [13], is commonly used in the food industry at concentrations of 0.02 to 0.08% (200-800 ppm) for equipment and utensil sanitation and up to 0.12% (1200 ppm) for facility cleaning [14]. This compound is highly, reacts with organic matter, and may irritate the skin, mucosa, and respiratory tracts of the manipulators [15]. Additionally, in environments with high presence of organic matter, its activity can be compromised because the available chlorine can be consumed, reducing the sanitizing capacity of the product. In addition, the pH must be maintained at 5 and 7 to ensure that the highest amount of acid hypochlorous acid is available [13]. Moreover, sodium hypochlorite needs attention in relation to its storage in order to avoid chlorate degradation, thus it must be kept in the dark and at mild temperatures to avoid loss of sanitizing activity [12].

The main sanitizers available in the market usually with authorized use by regulatory agencies and commonly used by the food industries worldwide are benzalkonium chloride, biguanide, peracetic acid, quaternary ammonium, and sodium hypochlorite, as well as other phenolic-compound based products [16, 17, 18].

Microbiota involved in the fungal spoilage of food products

The microbiota present in each food industry is influenced by the technological processes, raw materials, additives, and preservatives used, in addition to climatic

factors such as relative humidity and temperature. Some of the major fungi frequently reported as agents for the deterioration of bakery products, dairy products, and mature meat are described in Table 1.

Bakery product industry

Fungi are the main spoilage agents of bakery products due to product composition [rich in carbohydrates, moisture content (around 40%), water activity (aw 0.94 to 0.98), intermediate acidity (pH = 5.5–6.0)] [19] and the storage temperature these products are exposed to during commercialization (20–35 °C). These factors will result in the shelf life of 3–7 days for most bread that does not contain preservatives [20,21] and extends for up to 14 days for bread containing calcium propionate, which is the main preservative used in bakery products [22].

It is believed that all fungal spores present in flour are eliminated during bread baking and similar activities [23]. However, fungal spores from flour that disperse in the air as aerosols tend to settle on the surface of freshly baked products prior to being packaged, which is during the cooling stage. The ability to bypass the preservative barrier is the main determinant of the major spoilage species of industrialized bakery products.

The main filamentous fungi involved in contamination and consequent spoilage of bakery products are *Penicillium*, *Aspergillus, Wallemia, Fusarium,* and *Cladosporium* [24]. Likewise, yeast spoilage known as “chalky mold” is caused by *Hyphopichia* and *Endomyces* [25]. Among these genera, we highlight the species *Penicillium roqueforti*, *Penicillium paneum*, *Aspergillus pseudoglaucus* (formerly *Eurotium repens*), and *Hyphopichia burtonii* [22,25,26].

The known higher resistance of some species, such as *P. roqueforti*, compared to the main antifungal agent used by industry, which is calcium propionate [27], and its widespread occurrence in spoiled breads [22], demonstrates the need for the bakery industry to focus on preventive measures, which is restricted to the contact of the bread with the spores of these species. Therefore, choosing effective sanitizers for the problem fungus of each facility in particular is relevant, since certain fungal strains related to the spoilage of loaves have different resistance to different sanitizers [15]. Thus, control of contamination of the air and surfaces of equipment used during the industrial process, and especially decontamination of the cooling

environment, become essential in the fight against the early deterioration of this type of product.

Dairy industry

Soft and mature cheese is the most affected by fungi [28,27,29]. The fungal genera involved in the spoilage of these products originate mainly from the production environment, which includes the air, work surfaces, equipment, personnel, raw materials, and ingredients [30,31,32], in a similar way that has been mentioned in bakeries. As a result, the presence of fungi in the dairy industry is responsible for up to 5% of production loss [33].

Fungi and yeasts are the largest sources of dairy product spoilage. However, few studies have investigated fungal diversity in these products [20,32,34,35]. Among the 41 deteriorating fungal species isolated from dairy products analyzed by Garnier et al. [34], *P. commune* represented about 10% of the total. In another study, Kure et al. [30] found *P. roqueforti* ss. roqueforti in air samples and equipment of the dairy industry. In addition, *P. commune* was also found in samples of plastic film packages. Jahn et al. [32] also showed the presence of *P. commune* in the production air of a dairy industry having trouble with spoilage of a tropical-type cheese.

Industries have sought to control these microorganisms by adding antimicrobial compounds and preservatives during product manufacturing [36]. The most common preservatives used for controlling fungi and yeasts in dairy products are sorbic acid and its salts [37]. However, consumer demands for preservative-free products in recent years has increased, as healthier and safer food requirements have stipulated more natural ingredients in processed foods [33]. As a result, hygiene of the environment air and work surfaces has gained prominence.

Meat products industry

The air has been described as the main source of contamination in meat products [5,6,9], as its quality is often influenced by the quality of raw materials and environmental hygiene practices.

The genus *Penicillium* and *Aspergillus* are commonly isolated from cured meat products [7,9,38,39,40] and within these genera, some species, such as *Aspergillus westerdijkiae*, *Aspergillus ochraceus*, *Penicillium verrucosum*, and *Penicillium nordicum* are able to produce mycotoxins and, in this one in particular, ochratoxin A [41]. The presence of *A. ochraceus*/*A. westerdijkiae* has been reported on the surface of meat products [39,42,43]. Scaramuzza et al. [44] also reported the low quantity of this species in the air of 1 out of 3 meat processing plants in Italy. More recently, it was reported as a mycotoxin producer in cured ham and fermented sausages [40,45]. Due to the potential problem of the contamination of these products with mycotoxins, contamination prevention by careful cleaning of the environment of production and maturation is crucial [9].

In this sense, it is possible to see that hygienic control of the air inside of maturation chambers of cured meat products is one of the best ways to reduce the population of unwanted fungi. However, studies have shown that some fungal strains of meat products are very resistant to the sanitizers used [46].

Control of spoilage fungi by sanitizers

Even though food additives (in particular preservatives) should not be used to disguise flaws due to the failure to adopt good manufacturing practices; industries have sought to control these microorganisms by adding antimicrobial compounds and preservatives during product manufacturing [45,46], especially when an episode of fungal deterioration hits an industry. However, consumer demands for preservative-free products in recent years has increased, as healthier and safer food requirements have stipulated more natural ingredients in processed foods [33]; which sometimes reduces the products stability during storage and enhances the chance of a food spoilage episode by fungi to occur. As a result, hygiene of the environment (both air and work surfaces) has gained prominence, since it is one of the best ways to extend the shelf life by reducing the initial contamination of a certain food product.

In vitro sensitivity of spoilage fungi from food to sanitizers

The most widely used and known methods worldwide for evaluating the efficacy of sanitizers are the methods recommended by the Association of Official

Analytical Chemists (AOAC), which is based on the use of cylinders impregnated with reference microorganisms [47], and by the European Committee for Standardization (CEN), which recommends the use of stainless steel disks with 2 cm of diameter also impregnated with reference standard microorganisms [48]. Besides these methodologies generally used for liquid sanitizers, there is the French standard (Norme Française [NF] T-72281) for tests with air dispersed sanitizers that also use stainless steel disks and microorganism standards of reference [50].

According to the European Committee for Standardization (CEN) [48], a liquid sanitizer must reduce 3 log of the initial number of a fungal population (99.9% reduction) when compared with its respective positive control population in order to be considered effective. Regarding fumigant agents, there is a specific normative to be followed (Norme Française [NF] T-72281), which requires a reduction of 4 log (99.99% reduction) of the fungal population in order to prove their effectiveness [49].

Based on the *in vitro* antifungal efficacy results of sanitizers used in food industries obtained by Bernardi et al. [11,15,44,50], using the CEN and NF methodologies, a heatmap (Figure 1) was constructed in order to assist in choosing the most effective agents to control some problem fungi in the bakery, meat, and dairy industries. To produce this graph, the values of the reductions obtained at the highest concentrations recommended by the manufacturer on the product label were used [for the liquid sanitizers: benzalkonium chloride (5%), biguanide (5%), peracetic acid (3%), quaternary ammonium (5%), and sodium hypochlorite (0.2%); for the fumigant sanitizer: orthophenylphenol 15% ($1g/m^3$)], as well as using a seven-point scale of effectiveness [44].

The occurrence of sensitivity variation of strains isolated from the various spoilage products to the sanitizers commonly applied by the food industry was demonstrated. It can be due to the existence of phenotypic heterogeneity in the fungi evaluated, as well as adaptation to the selection pressure imposed by successive exposures to sanitizers in the industrial environment. In general, the peracetic acid was the sanitizer with the best antifungal activity among the chemical sanitizers evaluated [11,15,44,50]. Nevertheless, according to the heatmap, one can easily perceive that some fungal strains are resistant to all the sanitizers in the considered concentration.

The peracetic acid appears to exhibit its highest antifungal activity when confronted with spoilage fungi of bakery products. Its lowest efficacy occurs among strains isolated from cured meat products, especially *P. polonicum*.

Benzalkonium chloride has also proven to be a good agent when it comes to spoilage fungi of bakery products. Quaternary ammonia, which is a similar compound, obtained low efficacy fronts for the same isolates. However, it has shown considerable reductions in relation to spoilage cheese (*P. roqueforti* e *P. commune*). Bundgaard-Nielsen and Nielsen [51] also reported unsatisfactory results of ammonium quaternary compounds against *P. roqueforti*.

Orthophenylphenol-based fumigant was ineffective against most the species tested, however, this sanitizer achieved better results than the other sanitizers tested regarding *A. westerdijkiae* an important spoilage agent of cured meat products.

Sodium hypochlorite and biguanide rarely demonstrated adequate antifungal activity and were not considered good sanitizers for fungal control in the bread, cheese, and cured meat product industries at the evaluated concentrations.

The best results of sodium hypochlorite were achieved against the standard strains recommended for analyzing sanitizer antifungal efficacy. Unsatisfactory results of fungal control can be obtained in the food industry if just the sensitivity results of *A. brasiliensis* and *C. albicans* standard strains evaluations were used to choose the most appropriate sanitizer. Therefore, we highlight the importance of knowing the sensitivity of the strains present in the environment or spoiling the products elaborated in each food facility to choose the most suitable sanitizer for each specific situation.

In addition, the formation of biofilms by filamentous fungi is also an important factor to be considered when discussing environmental sanitation and factory production equipment. Some studies point to the fungus capacity of the genus *Aspergillus* (section Nigri and Flavi), *Penicillium*, *Cladosporium*, and *Alternaria* to form biofilms in aquatic environments and also on material surfaces in laboratory conditions [52]. Despite this, biofilms formed by yeast fungi of the genus *Candida* are still the most cited/documented/reported [53]. The presence of the extracellular matrix protecting fungal cells organized in biofilms against the action of sanitizers may be an additional challenge for fungal control in the food industry.

Conclusion

There is a diversity of fungi associated with different types of food products. Knowledge of species that may deteriorate each food group, sources of contamination, and dispersion methods of fungal particles are of extreme importance to choose the best forms of control. The air stands out as the main vehicle of fungal propagules dispersion in the food processing environment and diffuser of contamination between products. Reducing the number of contaminants in the processing environment by using chemical sanitizers is one of the best ways to prevent the problem of early food spoilage by fungi. Knowledge of the antifungal efficacy of the main sanitizing agents allowed in the food industry against the main spoilage fungi species of food can help in choosing the best agent for the industrial hygiene of each specific case and, therefore, collaborate in reducing losses caused by fungal spoilage.

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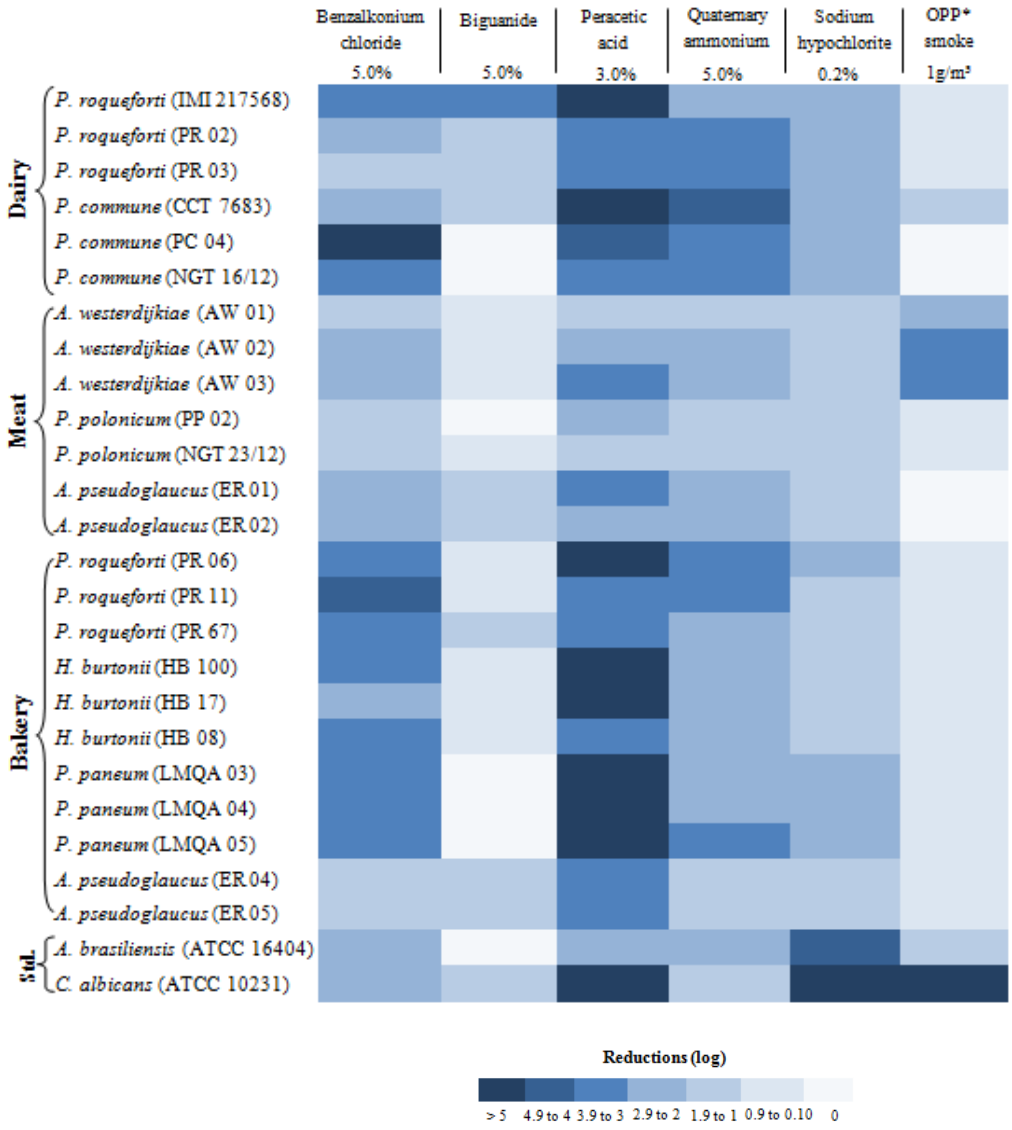


Figure 1. Heatmap of commercial sanitizers efficacy against cheese, cured meat and bakery product spoilage fungi based on the *in vitro* antifungal efficacy results obtained by Bernardi et al. [11,15,46,49] (*Orthophenylphenol).

Table 1. Intrinsic characteristics and contamination source of spoilage fungi according to the type of food industry.

Industry	Intrinsic characteristics of products	Spoilage fungi	Source of contamination	Source of problem
Bakery	Rich in carbohydrates, moisture content around 40%, water activity ~from 0.94 to 0.98, and intermediate acidity (pH = 5.5–6.0) [19].	*P. roqueforti*, *P. paneum*, *P. polonicum,* *P. citrinum*, *P. paxilli*, *P. glabrum*, *A. pseudoglaucus,* *A. flavus,* *A. sydowii,* *A. niger,* *C. sitophila* , *H.burtonii*, *E. fibuliger,* *W. sebi,* [22,24,25,26]	- Raw materials (cereals infected in the field by spores that remain viable in the flour); - Air of the processing environment.	- Fungal spores from the flours and dispersed in the air as aerosols; - Aerosols serve as a source of contamination when deposited on the surface of freshly baked products during the cooling stage (recontamination). - Ability to fungi bypass the preservative barrier.
Dairy	Rich in lipids, typical pH range 4.3-4.9, moisture around 30% hard cheese; 50% soft cheese; 50% margarines; 70 to 80% yogurts, water activity variable (0.93 to 0.98), redox potential [33].	*P. commune*, *P. roqueforti,* *P. polonicum*, *P. glabrum,* *P. chrysogenum,* *P. solitum,* *P. verrucosum,* *Aspergillus* sp., *C. famata*, *C. parapsilosis,* *M. hiemalis,* *G. candidum,* [30,31,32,33,34]	- Production environment: air, work surfaces, equipment, personnel, raw materials, and ingredients.	- Low hygienic level of the environment. - Spores dispersed in the air deposited on the surface of fresh products. - The air of maturation rooms.
Meat	Rich in proteins, carbohydrates, and fats. Typical final pH range (4.40-4.80), moisture range (30%-36%), water activity range (0.77-0.83) [45].	*A. westerdijkiae,* *A. ochraceus,* *A. candidus,* *P. polonicum,* *P. nordicum,* *P. verrucosum,* *P. nalgiovense,* *P. glabrum,* *C. cladosporioides,* *M. hiemalis,* *D. hansenii,* [7,9,38,39,40,41,42,43]	- Production environment: air, work surfaces, equipment, personnel, raw materials, and ingredients.	- Low hygienic level of the environment. - Spores dispersed in the air. - The environmental conditions of the maturation rooms. - Tolerance to low pH and high salt concentration.

7 DISCUSSÃO GERAL

É de vasto conhecimento entre os micologistas de alimentos que existem espécies de fungos comumente relacionadas a um nicho ecológico específico, o que pode culminar na deterioração de grupos de produtos alimentícios. Isto é determinado por uma interdependência de fatores fúngicos, características do alimento e condições de processamento (SNYDER et al., 2019). O conhecimento das espécies que podem deteriorar cada grupo de alimentos, as fontes de contaminação e os métodos de dispersão das partículas fúngicas são, portanto, de extrema importância para escolher as melhores formas de controle.

O ambiente de produção e/ou processamento, incluindo o ar, assumem um papel relevante na contaminação fúngica de um alimento, o que influenciará em sua estabilidade microbiológica, ou, relacionado ao objeto de estudo desta tese, no tempo até a ocorrência de deterioração fúngica. O tempo até a deterioração de um produto está relacionado ao nível da contaminação inicial, ou número de células fúngicas que entram em contato com um determinado alimento, sendo este maior quanto maior for o número de propágulos fúngicos dispersos no ar e ambiente de produção. Quando nos referimos à deterioração de produtos de panificação, é crucial considerar que esporos fúngicos oriundos das matérias-primas são dispersos no ar durante a elaboração do produto, contaminando o ambiente de produção, e poderão recontaminar a superfície destes produtos nas fases após o assamento (resfriamento, corte e embalagem). De maneira similar, produtos de laticínios e cárneos curados, como queijos e embutidos, que tem sua maturação em salas específicas tem seu tempo de vida útil influenciado pela micobiota que se estabelece nestes ambientes (*house flora*) e contaminam a superfície dos produtos assim que adrentam nestes ambientes (e que nem sempre são higienizadas entre uma batelada e outra). Com isso, reduzir o número de fungos no ambiente de produção e/ou processamento através da aplicação de sanitizantes é uma maneira promissora para prolongar a vida útil de alimentos e evitar o problema de deterioração precoce por fungos.

De acordo com o Comitê Europeu de Normalização quando um sanitizante líquido é capaz de reduzir 3 log (99,9%) de uma população fúngica inicial ele é considerado eficaz. Para agentes saneantes dispersados por via aérea e

preconizados pela Norma Francesa NF-T-72281 a redução fúngica necessária para garantir a eficácia é de 4 log (99,99%).

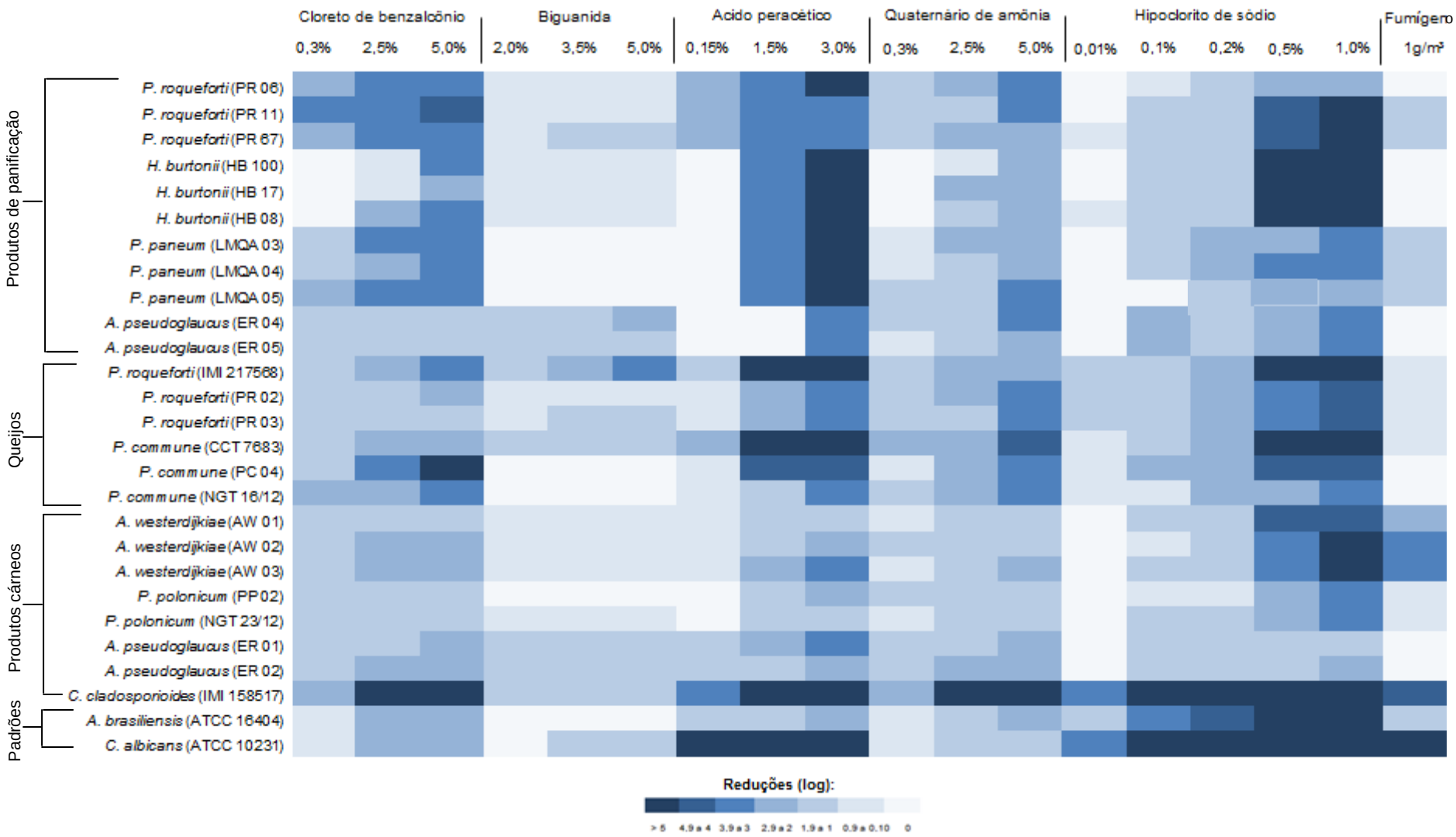
Baseado nos dados de ação antifúngica de sanitizantes obtidos com a execução deste projeto de doutoramento e apresentados nesta tese na forma de 5 artigos científicos, foi definida uma escala de eficácia de 7 pontos e construído um *heatmap* final (Figura 1) com o objetivo de facilitar a visualização dos resultados.

Dentre os sanitizantes testados no estudo, nenhum foi capaz de atender a legislação quando aplicadas as menores concentrações de uso indicadas no rótulo pelo fabricante. Com isso, se uma determinada indústria alimentícia optar por esta concentração, poderá não obter a eficácia desejada no processo de higienização; e não ser capaz de controlar o problema de deterioração precoce. Com o passar do tempo o nível de contaminação dos produtos poder aumentar e as perdas relacionadas persistirem.

De maneira geral, se considerarmos a média de concentrações dos sanitizantes testados, calculada a partir das máximas e mínimas indicadas no rótulo do produto pelo fabricante, chegaremos a algumas constatações que estão detalhadas a seguir.

Para as cepas de *P. roqueforti* PR 11 e PR 67 isoladas a partir de pães deteriorados a melhor opção de escolha de uso foi o cloreto de benzalcônio (2,5%) e ácido peracético (3%) e as piores escolhas, a biguanida, o quaternário de amônia (2,5%) e hipoclorito de sódio (0,1%), sendo que este composto também foi a pior escolha no caso da cepa de *P. roqueforti* PR 06. Por outro lado, a melhor redução obtida para a cepa PR 06 foi com testes com cloreto de benzalcônio e ácido peracético. Por outro lado, considerando ambas as cepas de *P. roqueforti* isoladas de pães, podemos dizer que o ácido peracético a 1,5% seria a melhor escolha, nesse caso, ao fato de ele possuir uma boa redução para ambas as cepas (PR 67, PR 11 e PR 06).

Figura 1: *Heatmap* da eficácia antifúngica de sanitizantes comerciais frente a fungos deteriorantes de interesse em alimentos.



Dentre as cepas de *H. burtonii* testadas a melhor escolha seria o ácido peracético e a pior seria a biguanida. Os resultados de boa eficácia em relação ao ácido peracético também podem ser observados para todas as cepas de *P. paneum* (LMQA 03, LMQA 04 e LMQA 05). O ácido peracético, além de seu princípio ativo, contém uma proporção de ácido acético em sua formulação. O ácido acético pode ser utilizado no pão agindo como um conservante, que reduz o pH da massa e melhora a ação do ácido propiônico, atuando como compostos antifúngicos (MARIN, et al., 2003).

As diferentes linhagens testadas da espécie *A. pseudoglaucus* (ER 04 e ER 05) isoladas de panetones deteriorados, foram extremamente resistentes aos sanitizantes utilizados nos testes, com exceção das concentrações mais altas de ácido peracético (3,0%) e hipoclorito de sódio (1,0%) que atingiram a "boa eficácia". Os demais agentes nas diferentes concentrações utilizadas nos testes atingiram apenas a classificação "baixa ou baixa eficácia" para ambas as cepas de *A. pseudoglaucus*. Da mesma maneira, as cepas de *A. pseudoglaucus* (ER 01 e ER 02) isoladas de produtos cárneos deteriorados também foram resistentes aos sanitizantes testados. Além disso, podemos dizer que demonstraram resistência ainda mais extrema em relação aos isolados de panetones, pois, somente obtiveram uma redução de 3 log para a concentração mais alta de ácido peracético (3,0%) e somente para a cepa de *A. pseudoglaucus* ER 01.

Podemos notar que houve variação de sensibilidade entre as cepas testadas de *P. roqueforti* isoladas a partir de queijos mofados (*P. roqueforti* PR 02 e PR 03), sendo que o quaternário de amônia (5%) se mostrou eficaz frente à cepa *P. roqueforti* PR 02 e seu derivado, o cloreto de benzalcônio se mostrou ineficiente para a cepa *P. roqueforti* PR 03 o que pode ser comparado, a *grosso modo*, com estudos realizados por Bundgaard-Nielsen e Nielsen (1996) que com uso de metodologia distinta obtiveram resultados insatisfatórios para cepas de *P. roqueforti*, além de os autores relatarem que compostos de quaternário de amônia foram ineficazes contra *P. roqueforti* (IET 11524). Em contrapartida, Korukluoglu et al. (2006) relataram que 5 cepas de *P. roqueforti* que testaram foram suscetíveis ao cloreto de benzalcônio e obtiveram inibição efetiva na concentração de 2% entre 2 e 11 min de exposição, na qual a cepa do queijo era uma das mais sensíveis.

Para as cepas de *P. commune* (PC 04, CCT 7638 e NGT 16/12) foi possível observar uma maior linearidade nos resultados encontrados, sendo que a maioria

dos sanitizantes apresentaram resultados satisfatórios, com exceção da biguanida (3,5%) que foi ineficaz contra as cepas de *P. commune* PC 04 e NGT 16/12.

Mais uma vez, destacamos a importância de se conhecer a sensibilidade das cepas presentes no ambiente em cada indústria de alimentos para escolher o sanitizante mais adequado. Além disso, a maior sensibilidade apresentada pelas cepas adquiridas de um banco de culturas, *P. roqueforti* (IMI 217568) e *P. commune* (CCT 7683) ilustra que pode haver diferença no resultado esperado se para análise da eficácia de sanitizantes forem selecionadas cepas de coleções de cultura e não aquelas responsáveis por problemas de deterioração na indústria alimentícia.

Já em relação aos isolados fúngicos de produtos cárneos deteriorados, a presença de *A. westerdijkiae* apresenta um risco em relação à produção de ocratoxina A, considerada a micotoxina mais relevante nesses produtos (PARUSSOLO et al., 2019b). Nesse sentido, a sanitização do ar e ambiente de câmaras de maturação de produtos cárneos curados é importante para reduzir a contaminação inicial e prevenir o desenvolvimento de fungos indesejados no produto. Contudo os estudos demonstraram que cepas de *A. westerdijkiae* AW 01, AW 02 e AW 03, isoladas a partir de salames deteriorados, se mostraram muito resistentes aos sanitizantes testados. Resultados similares de resistência foram observados para as cepas de *P. polonicum* (PP 02 e NGT 23/12). Assim, as cepas fúngicas isoladas de produtos cárneos deteriorados destacam-se como as de maior resistência dentre as avaliadas neste estudo. Em situações como esta, uma alternativa poderia ser utilizado o composto fumigante à base de ortofenilfenol, que embora não tenha sido capaz de reduzir os 4 log (99,99%) exigidos pela norma francesa, conseguiu uma redução de 3 log (99,9%). Adicionalmente, em situações críticas poderiam ser extrapoladas as concentrações recomendadas. Apesar da corrosividade de altas concentrações de hipoclorito de sódio, testamos concentrações altas, visto que este é um composto de uso comum em indústrias alimentícias. Concentrações de 0,5% (5000 ppm) foram eficazes contra os isolados de *A. westerdijkiae,* porém foram necessárias concentrações de 1% (10000 ppm) para redução dos *P. polonicum* à níveis satisfatórios.

Uma prática que se observa em algumas fábricas de queijos e embutidos curados é a lavagem do produto mofado para remoção do micélio fúngico visível (PARUSSOLO et al., 2019a; IACUMIN et al., 2009) e assim reduzir a contaminação do produto com micotoxinas, apesar de ocorrer a difusão de parte das micotoxinas

ao centro do produto (COTON, et al., 2019; PARUSSOLO et al., 2019b). Do ponto de vista higiênico, esta prática também é bastante preocupante, visto que durante a lavagem dos produtos, uma alta carga de esporos fúngicos é liberada no ar, elevando a contaminação do ambiente (PARUSSOLO et al., 2019a). Estes esporos provenientes de produtos visivelmente mofados (deteriorados) já estariam adaptados ao substrato e poderiam atuar como uma espécie de inóculo inicial, sendo o ambiente a fonte de contaminação para produtos recém-dispostos para maturação nas câmaras, podendo dominar rapidaemente a superfície dos produtos, culminando em deterioração precoce em lotes subsequentes.

Por fim, os resultados quanto à eficácia dos sanitizantes frente aos fungos padrões para este tipo de teste, *C. albicans* ATCC 10231 e *A. brasiliensis* ATCC 16404, foram bastante divergentes dos resultados de sensibilidade apresentados pelos fungos-problema avaliados neste estudo. O hipoclorito de sódio, por exemplo, só foi eficaz nas concentrações mais baixas (0,01% e 0,1%) contra as cepas padrões para testes recomendadas pelo CEN e NF.

Ademais, a *C. albicans* ATCC 10231 foi a mais sensível de ambos os padrões, obtendo reduções de 3 log para maioria dos sanitizantes nas concentrações intermediárias e máximas testadas, com exceção da biguanida. Portanto, se uma indústria levar em consideração apenas estas informações “padrão” para a escolha do agente sanitizante e concentração a ser empregada, resultados insatisfatórios de controle fúngico podem ser obtidos.

Em suma, partindo-se da premissa do desconhecimento da espécie fúngica deteriorante a ser combatida em uma determinada indústria alimentícia, recomendaria-se:

-caso se trate de indústria de panificação, onde se utilize conservantes como propionato de cálcio, para a higienização do ambiente seria recomendado o uso de concentrações intermediárias ou máximas de ácido peracético e/ou as máximas de cloreto de benzalcônio no programa de higienização;

-em indústrias de queijos, o sanitizante de eleição seria o ácido peracético na concentração máxima, porém resultados satisfatórios também poderiam ser obtidos com amônia quaternária;

-em indústrias de processados cárneos (salames e outros embutidos fermentados, carnes maturadas, nuggets etc.) poderá ser necessária a aplicação de sanitizantes em concentrações superiores ao recomendado pelo fabricante, e

resultados satisfatórios talvez sejam também obtidos com a utilização de sanitizante fumigante à base de ortofenilfenol.

Assim, o conhecimento da eficácia antifúngica dos principais agentes sanitizantes permitidos na indústria alimentícia frente às principais espécies de fungos deteriorantes de alimentos pode auxiliar na escolha do melhor agente para a higiene industrial de cada caso específico e, portanto, colaborar na redução de perdas econômicas por deterioração fúngica de alimentos.

8 CONCLUSÃO GERAL

Segundo os resultados *in vitro*, as concentrações mais baixas recomendadas pelo fabricante são na maioria das vezes ineficazes para a eliminação de fungos deteriorantes nos níveis exigidos pela legislação (>99,9% de redução).

Foi demonstrada a existência de variação de sensibilidade entre as cepas de fungos isoladas de vários produtos alimentícios deteriorados frente à sanitizantes comumente aplicados em indústrias de alimentos, sendo observada também divergência nos resultados de eficácia quando comparadas estas com as cepas padrões para este tipo de teste.

Em geral o ácido peracético foi o sanitizante de maior eficácia, sendo considerado o melhor princípio ativo entre todos os testados. Contudo, pudemos observar que algumas cepas foram resistentes a todos os sanitizantes.

O ácido peracético parece demonstrar sua maior eficácia quando confrontado com as cepas oriundas de produtos panificados e em oposição uma eficácia mais reduzida quando relacionado com as cepas de produtos cárneos curados, especialmente com *P. polonicum* e *A. westerdjikae.*

O cloreto de benzalcônio também se mostrou um bom agente quando se trata de fungos isolados de produtos panificados, podendo ser utilizado alternado ao ácido peracético. O quaternário de amônia, por outro lado, apresentou baixa eficácia frente aos mesmos deteriorantes, contudo demonstrou ser um bom composto quando de trata de controle de fungos deteriorantes de queijos.

O hipoclorito de sódio, a biguanida e o agente fumigante a base de ortofenilfenol raramente apresentaram eficácia satisfatória frente aos fungos testados, podendo não ser adequados para uso em indústrias alimentícias.

A elaboração de escalas antifúngicas de 5 e 7 pontos facilitou a visualização dos resultados de eficácia dos sanitizantes, sendo adequada para processos decisórios envolvendo número elevado de cepas fúngicas e/ou sanitizantes em variadas concentrações.

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APÊNDICE A – AUTORIZAÇÃO PARA DIVULGAÇÃO DE ARTIGO

APÊNDICE B – AUTORIZAÇÃO PARA DIVULGAÇÃO DE ARTIGO

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APÊNDICE D – AUTORIZAÇÃO PARA DIVULGAÇÃO DE ARTIGO

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ELSEVIER

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Author: Angélica Olivier Bernardi,Marcelo Valle Garcia,Marina Venturini Copetti

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