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COMO AGENTES DE CONTROLE DE FUNGOS TOXIGÊNICOS

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FLÁVIO FONSECA VERAS

Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul – UFRGS como um dos requisitos para a obtenção do Grau de Doutor em Ciência e Tecnologia de Alimentos.

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William Shakespeare

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RESUMO

Fungos filamentosos com capacidade de produzir micotoxinas podem estar presentes em alimentos, desde o cultivo até o produto após industrialização. Devido a isso, estratégias para controlar o crescimento fúngico devem ser investigadas, a fim de evitar o desenvolvimento desses microrganismos, bem como a produção de suas toxinas nos alimentos. Neste trabalho, duas abordagens para o controle de fungos toxigênicos foram avaliadas. A primeira estratégia foi a utilização de bactérias provenientes de diferentes ambientes aquáticos, sendo que 10 linhagens de *Bacillus* spp. e a linhagem *Pseudomonas* sp. 4B foram testadas quanto à influência sobre os parâmetros de crescimento (taxas de crescimento micelial, esporulação e germinação de esporos) de fungos toxigênicos (*Aspergillus* e *Penicillium*) e formação de micotoxinas. Todas as bactérias foram capazes de inibir o crescimento dos fungos em meio de cultura, apresentando halos de inibição variando de 1,0 até 15,7 mm. *Bacillus* sp. P11 apresentou resultados mais expressivos em relação às demais linhagens do gênero *Bacillus* com maiores valores de redução na maioria dos parâmetros de crescimento. Além disso, *Bacillus* sp. P11 e *Pseudomonas* sp. 4B apresentaram efeito sobre as taxas de crescimento micelial, esporulação e germinação de esporos, com níveis de redução acima de 43,3, 32,1 e 84,1% respectivamente. Mesmo assim, as demais linhagens também apresentaram resultados satisfatórios sobre esses parâmetros. Estas bactérias também reduziram a síntese de aflatoxina B1 e ocratoxina A em mais de 94 e 63%, respectivamente, quando cultivadas simultaneamente com os fungos produtores de cada micotoxina. Adicionalmente, a capacidade de *Bacillus* sp. P11 em produzir os lipopeptídeos iturina A (167,9 mg/mL de extrato butanólico) e surfactina (361,9 mg/mL de extrato butanólico) foi confirmada. Estes compostos podem ter contribuído para a atividade antifúngica desta bactéria. A segunda estratégia investigada neste estudo para controlar o desenvolvimento de fungos toxigênicos foi o emprego de nanofibras de poli- ϵ -caprolactona (PCL) incorporadas com cetoconazol e natamicina como material antimicrobiano. Nesta abordagem, as nanofibras foram produzidas pela técnica de eletrospinning e posteriormente caracterizadas e avaliadas quanto ao seu potencial antifúngico. Nanofibras funcionalizadas com cetoconazol ou natamicina apresentaram atividade antifúngica contra os isolados toxigênicos uma vez que zonas de inibição variando de 6 a 44 mm foram observadas. Além disso, as análises de microscopia eletrônica e espectroscopia demonstraram que a incorporação dos antifúngicos não altera de forma expressiva as principais características das nanofibras. Também foi possível verificar a capacidade de liberação controlada dos antifúngicos durante 72 h de contato das nanofibras com diferentes soluções simulantes. Valores próximos a 80 e 45 μ g/mL de cetoconazol e natamicina, respectivamente, foram observados em solução de Tween 20 (5%). Portanto, o processo de eletrospinning foi capaz de agregar propriedades antifúngicas às nanofibras de PCL. Os resultados demonstraram que as bactérias e os nanomateriais investigados neste estudo são promissores para o controle de fungos toxigênicos e produção de micotoxinas.

Palavras-chave: Micotoxinas, agentes antifúngicos, controle biológico, nanotecnologia, nanomateriais

ABSTRACT

Filamentous fungi that have the potential to produce mycotoxins may be present in food, from cultivation to after industrialization. Therefore, several strategies to control fungal growth must be investigated in order to avoid the development of these microorganisms and the production of their toxins in food. In this work, two approaches to toxigenic fungi control were evaluated. The first one was the use of bacteria from different aquatic environments as biocontrol agents in which 10 *Bacillus* spp. strains and the *Pseudomonas* sp. 4B strain were tested in relation to the effect on growth parameters (mycelial growth, sporulation and spore germination rates) of toxigenic fungi (*Aspergillus* and *Penicillium*) and mycotoxin formation. All bacteria were able to inhibit the fungal growth in culture medium with inhibition zones ranging from 1.0 to 15.7 mm. It was also observed that *Bacillus* sp. P11 had better results compared to other *Bacillus* strains with larger reduction values in most of growth parameters. Furthermore, *Bacillus* sp. P11 and *Pseudomonas* sp. 4B exhibited effect on mycelial growth, sporulation and spore germination rates with reduction values above of 43.3, 32.1 and 84.1%, respectively. Even so, the other strains also showed satisfactory results on these parameters. Finally, these bacteria reduced the synthesis of aflatoxin B1 and ochratoxin A at levels above 94 and 63%, respectively, when co-cultivated with each mycotoxin producing fungi. Additionally, the ability of *Bacillus* sp. P11 to produce lipopeptides such as iturin A (167.9 mg/ml of butanolic extract) and surfactin (361.9 mg/ml of butanolic extract) was confirmed. These compounds may have contributed to antifungal activity of this bacterium. The second investigation of this work in order to control the growth of toxigenic fungi was the use of poly- ϵ -caprolactone nanofibers incorporated with ketoconazole and natamycin as antimicrobial material. In this approach, nanofibers were produced by the electrospinning technique and subsequently characterized and evaluated for their antifungal potential. Both nanofibers functionalized with ketoconazole and natamycin showed antifungal activity against toxigenic isolates since inhibitory zones ranging from 6 to 44 mm were observed. In addition, scanning electron microscopy and infrared spectroscopy analysis showed that the antifungals incorporation does not change the characteristics of nanofibers. It was also possible to verify the ability of controlled drug release during 72 h of nanofibers contact with different simulants solutions. Values near 80 and 45 μ g/ml of ketoconazole and natamycin, respectively, were observed in the solution containing 5% Tween 20. Therefore, the electrospinning process was able to provide antifungal properties to the nanofibers. The results showed that bacteria and nanomaterials investigated in this study are promising for developing control strategies of toxigenic fungi and mycotoxin production.

Keywords: Mycotoxins, antifungal agents, biocontrol, nanotechnology, nanomaterials

INTRODUÇÃO

A produção e exportação de produtos agropecuários vêm exigindo um rigoroso controle de qualidade na produção, armazenamento, transporte e distribuição dos mesmos, a fim de serem observados os padrões sanitários em vigor, objetivando a preservação da saúde humana e animal. No entanto, a presença de substâncias nocivas em alimentos, mesmo que em pequenas concentrações e quando consumidas por períodos prolongados pode proporcionar sérios prejuízos à saúde, como é o caso da contaminação dos alimentos por micotoxinas.

O termo micotoxina refere-se a um grupo diversificado de substâncias quimicamente tóxicas, com diferentes fórmulas estruturais, propriedades químicas, físicas e toxicológicas, tendo em comum apenas o fato de serem metabólitos secundários obtidos através da biossíntese fúngica. Representantes dos gêneros *Aspergillus*, *Penicillium* e *Fusarium* estão entre os fungos toxigênicos mais importantes e frequentemente relatados na literatura. Dentre as micotoxinas de maior significância em produtos alimentícios estão as aflatoxinas e a ocratoxina A, que se destacam pela maior toxicidade e propriedades carcinogênicas, mutagênicas e teratogênicas. Por outro lado, algumas micotoxinas deveriam receber uma maior atenção devido a sua co-ocorrência com outras, como é o caso da citrinina, encontrada frequentemente associada à ocratoxina A. Estas micotoxinas podem agir sinergicamente aumentando a probabilidade de ocorrer efeitos nefrotóxicos.

A esses problemas de saúde acrescentam-se frequentes perdas econômicas nos setores das cadeias produtivas. Estudos demonstram que aproximadamente 25 a 50% das matérias-primas produzidas no mundo, estão contaminadas com fungos, sendo rejeitadas pelos mercados consumidores, interferindo ou impedindo a exportação, reduzindo a produção agrícola e animal ou aumentando seu custo.

Métodos químicos, físicos e biológicos têm sido investigados como estratégias para evitar a exposição às micotoxinas. No entanto, a utilização de alguns destes métodos pode implicar em algumas limitações como alto custo e complexidade para a detoxificação dos alimentos, além da ineficiência para grandes lotes ou até mesmo a necessidade de estudos mais aprofundados. Diante disto, a melhor forma de evitar a contaminação de micotoxinas nos alimentos continua sendo a prevenção de sua

síntese, principalmente através da inibição do crescimento fúngico em todas as etapas da cadeia de produção de alimentos.

No que se refere ao controle da contaminação fúngica de produtos agrícolas, embora o uso de agroquímicos seja reconhecido como um tratamento eficaz, há uma necessidade de encontrar meios seguros de controlar estes patógenos devido à toxicidade dos resíduos de fungicidas sintéticos para a saúde humana e o ambiente. Desta forma, a aplicação de agentes biológicos, bem como a utilização de nanomateriais, podem ser consideradas ferramentas promissoras para reduzir ou eliminar o uso de agroquímicos no controle de fungos, devido às várias possibilidades de combater o crescimento fúngico.

Entre as opções mais interessantes para serem usadas como agentes de controle biológico estão as bactérias dos gêneros *Bacillus* e *Pseudomonas*, que têm sido intensamente investigadas devido à produção de um grande número de substâncias com ação antimicrobiana, como lipopeptídeos e enzimas. Estes compostos têm demonstrado potencial para inibir o crescimento de fungos e conseqüentemente a produção de micotoxinas. Recentemente, linhagens de *Bacillus* provenientes de ambiente aquático da Região Amazônica apresentaram atividade antimicrobiana contra bactérias, vírus e fungos não toxigênicos. Da mesma forma, *Pseudomonas* sp. 4B isolada das águas de tratamento de efluentes de um frigorífico da região do vale do Rio Pardo, Rio Grande do Sul, também demonstrou efeito sobre bactérias e leveduras patogênicas e deteriorantes de alimentos. No entanto não há relatos específicos da utilização desses isolados bacterianos contra fungos produtores de micotoxinas, logo o potencial dessas bactérias para o controle do crescimento de fungos toxigênicos deve ser investigado.

Convém ressaltar que o controle de micotoxinas em alimentos não deve ser associado exclusivamente à busca por alternativas para evitar a contaminação fúngica da matéria-prima durante a pré-colheita. Na indústria de alimentos, por exemplo, falhas durante o processamento ou a falta de monitoramento totalmente efetivo das condições ambientais podem favorecer o desenvolvimento de uma microbiota indesejável no produto final, incluindo a presença de fungos toxigênicos, causando também perdas econômicas ou ainda rejeição pelo consumidor. Neste caso, estratégias de controle microbiano na indústria também devem ser investigadas, porém compreendendo outras abordagens tecnológicas com a finalidade de melhorar

a qualidade e segurança dos alimentos, como por exemplo o emprego de ferramentas nanotecnológicas. Esta nova área consiste no desenvolvimento, manipulação e caracterização de estruturas e materiais em escala nanométrica. As diferentes propriedades desses materiais permitem novas funcionalidades para inúmeras aplicações em alimentos, incluindo o controle de microrganismos e seus metabólitos.

Entre as nanoestruturas com grande potencial antimicrobiano, as nanofibras poliméricas obtidas pela técnica de *electrospinning* têm recebido enorme atenção devido a sua simplicidade e baixo custo de produção, e principalmente pela capacidade de incorporação de diferentes compostos com posterior liberação controlada, adquirindo função específica correspondente à substância adicionada. Vários materiais podem compor a matriz das nanofibras, incluindo a poli- ϵ -caprolactona (PCL), um polímero sintético, biodegradável e de baixa toxicidade, que vem sendo bastante favorável para incorporação de compostos pouco solúveis em água. Apesar dos vários atributos adequados para o desenvolvimento de nanofibras funcionalizadas com antifúngicos, poucos estudos têm sido realizados com esta finalidade (independente da matriz polimérica).

Visando futuras pesquisas sobre a elaboração de nanofibras incorporadas com os possíveis metabólitos das bactérias previamente citadas, estudos prévios utilizando antifúngicos comerciais em nanofibras de PCL também são necessários para verificar a funcionalização destes nanomateriais. Compostos como o cetoconazol e a natamicina, por exemplo, se destacam como antifúngicos de amplo espectro de ação, uma vez que causam efeitos negativos na membrana celular dos fungos. O cetoconazol pode ser utilizado em várias formulações farmacêuticas de uso tópico e sistêmico, enquanto a natamicina é comumente empregada em alimentos como aditivo antifúngico. Uma vez que são compostos de natureza hidrofóbica, sua incorporação em nanofibras de PCL poderia favorecer uma ação antifúngica mais efetiva devido a melhoria na distribuição desses fármacos e redução da quantidade utilizada. Neste contexto, a incorporação destes antifúngicos em nanofibras de PCL, a caracterização dessas estruturas e seu efeito *in vitro* contra fungos também são de extrema importância.

Diante do exposto, este estudo abordou duas estratégias para o controle de fungos toxigênicos: i) a utilização de bactérias dos gêneros *Bacillus* e *Pseudomonas* como agentes de controle biológico; e ii) o emprego de nanofibras de poli- ϵ -

caprolactona funcionalizadas com os antifúngicos cetoconazol e natamicina como possível material antimicrobiano para liberação controlada dos respectivos fármacos.

OBJETIVOS

Objetivo geral

Avaliar o potencial de utilização de bactérias dos gêneros *Bacillus* e *Pseudomonas*, bem como de nanofibras funcionalizadas com compostos antifúngicos, como agentes de prevenção da contaminação por fungos produtores de micotoxinas.

Objetivos específicos

- Investigar a atividade antagonista de *Bacillus* spp. provenientes da Bacia Amazônica contra fungos toxigênicos e posteriormente selecionar o(s) melhor(es) representante(s);
- Verificar o potencial antagônico de *Pseudomonas* sp. 4B (isolada de um lago de tratamento de efluentes) no controle de fungos produtores de micotoxinas;
- Avaliar o efeito de *Bacillus* spp. e *Pseudomonas* sp. 4B sobre a produção de micotoxinas;
- Identificar o(s) componente(s) antimicrobiano(s) produzido(s) pelas bactérias;
- Verificar a eficácia de incorporação de cetoconazol, bem como da natamicina, durante o desenvolvimento de nanofibras de poli- ϵ -caprolactona pela técnica de *electrospinning*;
- Caracterizar e comparar as nanofibras através de análises microscópicas, espectrofotométricas e termogravimétricas;
- Verificar a capacidade de liberação controlada dos antifúngicos incorporados nas nanofibras em soluções simulantes.

CAPÍTULO 1

1. REVISÃO DE LITERATURA

1.1 Fungos toxigênicos e micotoxinas

Muitos dos fungos que se desenvolvem nos alimentos produzem substâncias tóxicas quando existem fatores biológicos e ambientais adequados. Do ponto de vista do metabolismo dos fungos, são considerados metabólitos primários os compostos essenciais para seu crescimento, tais como proteínas, lipídeos, ácidos nucleicos e carboidratos, enquanto que os metabólitos secundários, como as micotoxinas, não têm qualquer significado bioquímico no crescimento e desenvolvimento fúngico (SCUSSEL, 1998; MURPHY et al., 2006; ROCHA et al., 2014).

Está bem estabelecido que nem todos os metabólitos secundários fúngicos são tóxicos, e nem todos os fungos são toxigênicos. Além disso, um mesmo fungo pode produzir simultaneamente diferentes tipos de micotoxinas (BRAGULAT et al., 2008; IAMANAKA et al., 2010; ROCHA et al., 2014).

É difícil definir micotoxinas em poucas palavras, no entanto o termo pode ser conceituado como um grupo de compostos heterogêneos, em sua maioria de baixa massa molecular, produzidos pelo metabolismo secundário de alguns fungos filamentosos que, quando ingeridos, inalados ou absorvidos podem causar doenças ou morte (PITT, 2000; MARROQUIN CARDONA et al., 2014). Tais compostos pertencem a diferentes grupos químicos (pironas, antraquinonas, cumarinas, macrolídeos, esteroides, polipeptídeos cíclicos, etc.) (WEIDENBORNER, 2001; BENNET; KLICH, 2003; MARIN et al., 2013).

As micotoxinas têm causado grandes epidemias no homem e animais domésticos. Há especulações desde a Idade Média de vários surtos atribuídos ao consumo de alimentos preparados a partir de grãos contaminados (EDWARDS et al., 2002; SHERIFF et al., 2009). No entanto, a história das micotoxinas teve seu início registrado apenas nos anos 60, com a identificação das aflatoxinas após um incidente na Inglaterra com a morte de mais de 100.000 perus. A partir de então pesquisas sobre métodos de análises, estruturas químicas, efeitos toxicológicos e identificação

de organismos produtores expandiram de forma exponencial (BENNETT; KLICH, 2003; KIM et al., 2011; ZAIN, 2011; MARIN et al., 2013; RUBERT et al., 2013; SCAGLIONI et al., 2014; BOLECHOVÁ et al., 2015).

Pelo menos cerca de 400 compostos já foram reconhecidos como micotoxinas (ZINEDINE; MAÑES, 2009; BRYDEN, 2012), no entanto, as de maior significância são as aflatoxinas, ocratoxinas, fumonisinas, tricotecenos e zearalenona (KUMAR et al., 2008; SANTOS et al., 2009; MARIN et al., 2013; Ferre, 2016). Estas toxinas se destacam não só pela ampla ocorrência, mas principalmente devido à sua maior toxicidade, apresentando efeitos nefrotóxicos, hepatotóxicos, neurotóxicos e imunotóxicos, além de propriedades carcinogênicas, mutagênicas e teratogênicas (HUSSEIN; BRASEL, 2001; CREPPY, 2002; ROCHA et al., 2014).

A Agência Internacional de Pesquisa sobre o Câncer (do inglês: *International Agency for Research on Cancer*, IARC) avaliou o potencial carcinogênico destes compostos (IARC, 1993, 2012) e classificou as aflatoxinas como agentes carcinogênicos para humanos (Grupo 1), enquanto a ocratoxina A está classificada no Grupo 2B, entre os possíveis agentes carcinógenos aos humanos. Os riscos das micotoxinas para a saúde humana ou animal têm sido examinados extensivamente nos últimos anos (MARIN et al., 2013; ROCHA et al., 2014; SILVA et al., 2015; BOL et al., 2016).

Os efeitos tóxicos causados pelas micotoxinas variam de acordo com a micotoxina em questão. Enquanto algumas causam danos funcionais ao fígado e rim, outras se apresentam neurotóxicas ou interferem na síntese de proteínas, causando efeitos relacionados à sensibilidade cutânea e até mesmo ocasionando imunodeficiência extrema (MARIN et al., 2013; ROCHA et al., 2014). Além disso, o grau desses efeitos (agudos e/ou crônicos) são influenciados de acordo com o nível de exposição (HUSSEIN; BRASEL, 2001; STOEV, 2015).

Além dos problemas de saúde acrescentam-se perdas econômicas importantes nos setores das cadeias produtivas (SANTURIO, 2000; KUMAR et al., 2008; MARIN et al., 2013; AMARE; KELLER, 2014). As micotoxinas afetam o agronegócio de muitos países, dificultando a exportação, reduzindo a produção animal e agrícola ou ainda elevando seu custo (FREIRE et al., 2007). Oliveira et al. (2014) comentam, por exemplo, que o prejuízo estimado para cereais contaminados com fungos e

micotoxinas, além das despesas equivalentes à prevenção e descontaminação corresponde a bilhões de euros por ano. Além disso, outros estudos demonstram que mais de 25% das matérias-primas produzidas no mundo, estão contaminadas com fungos toxigênicos e micotoxinas, sendo rejeitadas pelos mercados consumidores (FREIRE et al., 2007; SEKIYAMA et al., 2007; FAO, 2013). Como os produtos de boa qualidade são normalmente exportados, aqueles que apresentam níveis de toxinas fúngicas superiores aos permitidos nos países importadores podem ser vendidos e consumidos no mercado interno (quando não há legislação própria), aumentando os riscos para a saúde da população dos países em desenvolvimento (FREIRE et al., 2007).

A contaminação de alimentos por micotoxinas tem sido relatada em todo o mundo, principalmente em alimentos suscetíveis ao crescimento fúngico, como cereais e frutas (PITT; HOCKING, 2009; OLIVEIRA et al., 2014). Representantes dos gêneros *Aspergillus*, *Penicillium* e *Fusarium* estão entre os fungos toxigênicos mais importantes e frequentemente relatados na literatura devido à grande ocorrência em alimentos e ao maior potencial de produção de micotoxinas (SWEENEY; DOBSON, 1998; CAST, 2003; SIMAS et al., 2007; RODRIGUES et al., 2012; HAAS et al., 2013; AHMAD et al., 2014; KONG et al.; 2014). A Tabela 1 apresenta as principais espécies de fungos toxigênicos e suas micotoxinas.

Tabela 1 - Principais micotoxinas e fungos toxigênicos encontrados em alimentos

Micotoxina	Espécies produtoras	Alimentos
Aflatoxinas	<i>Aspergillus</i> seção <i>Flavi</i> <i>Aspergillus</i> seção <i>Circumdati</i>	Amendoim, milho uva, café,
Ocratoxina A	<i>Aspergillus</i> seção <i>Nigri</i> <i>Penicillium verrucosum</i> e <i>P. nordicum</i>	Arroz, trigo, feijão, castanha, nozes
Fumonisinias	<i>Fusarium</i> seção <i>Liseola</i>	Milho, aveia, cevada
Tricotecenos	<i>Fusarium acuminatum</i> , <i>F. poae</i> , <i>F. sporotrichioides</i> , <i>F. langsethiae</i>	Trigo, cevada, milho
Zearalenona	<i>F. graminearum</i> , <i>F. equiseti</i> , <i>F. cerealis</i>	Milho, trigo, aveia

Fonte: Marin et al. (2013), adaptado

Fatores como concentração de fungos presentes no material, atividade de água, aeração e temperatura do substrato, interações microbianas, danos mecânicos, infestação por insetos e suscetibilidade de certas espécies vegetais em serem contaminadas por fungos estão relacionados às condições adequadas na produção de toxinas (CALVO et al., 2002; RODRÍGUEZ-AMAYA; SABINO, 2002; BENNETT; KLICH, 2003).

Vale ressaltar que os fatores biológicos, físicos e químicos podem atuar de modo isolado, porém, na maioria dos casos observa-se um efeito conjunto sobre o crescimento fúngico e a produção de micotoxinas (CALVO et al., 2002). Além disso, nem sempre as condições favoráveis à infestação coincidem com a produção de micotoxinas, contudo, uma vez produzidas permanecem após a morte do fungo produtor, mesmo quando este é substituído por outros microrganismos. A presença de um fungo não significa necessariamente que a micotoxina esteja presente ou vice-versa (PEREIRA et al., 2002; BENNETT; KLICH, 2003; ZAIN, 2011; COPETTI et al., 2013).

A produção de micotoxinas em culturas agrícolas pode ocorrer em vários pontos da cadeia alimentar: na pré-colheita, colheita, secagem e armazenamento (SFORZA et al., 2006; MARIN et al., 2013). A exposição às micotoxinas, pode resultar principalmente da ingestão de alimentos contaminados com as toxinas (exposição direta) ou ainda pelo consumo de produtos animais contendo quantidades residuais de micotoxinas, como leite, carne e ovos (exposição indireta) (CAST, 2003). Raramente ocorrem casos de exposição por outras vias tais como a inalação e contato direto com a pele (JARVIS, 2002).

1.2 Aflatoxinas

As aflatoxinas são um grupo de substâncias estruturalmente semelhantes, produzidas, sobretudo por *Aspergillus flavus* e *A. parasiticus*. Outras espécies produtoras, embora menos comuns, são *A. nomius*, *A. pseudotamarii*, *A. bombysis*, *A. parvisclerotigenus*, *A. ochraceoroseus*, *A. rambellii*, *A. pseudocaelatus*, *A. pseudonomius*, *Emericella venezuelensis*, *E. astellata*, *Aschersonia coffeae* e *As.*

marginata (FRISVAD et al., 2005; KLICH, 2007; VARGA et al., 2011; MASSI et al., 2014; BARANYI et al., 2015; VARGA et al., 2015).

Este grupo de micotoxinas é o que teve maior impacto em pesquisas na área da micotoxicologia, devido ao incidente ocorrido na Inglaterra com a morte de mais de 100.000 aves alimentadas com ração contaminada procedente do Brasil e da África (surto conhecido como doença X do peru) durante a década de 60 (COLE; COX, 1981). Tempos depois, uma substância tóxica produzida por um fungo que foi identificado como sendo *Aspergillus flavus*, possuía as mesmas propriedades tóxicas da ração relacionada ao surto, por conta disso o nome aflatoxina foi atribuído a esse composto (PITT; HOCKING, 2009; BARANYI et al., 2015; VARGA et al., 2015). Estruturalmente, as aflatoxinas pertencem à classe de compostos denominados furanocumarinas, diidrofuranos ligados a um anel cumarínico, que por sua vez, está ligado a um anel ciclopentona ou lactona (Figura 1) (BARANYI et al., 2015).

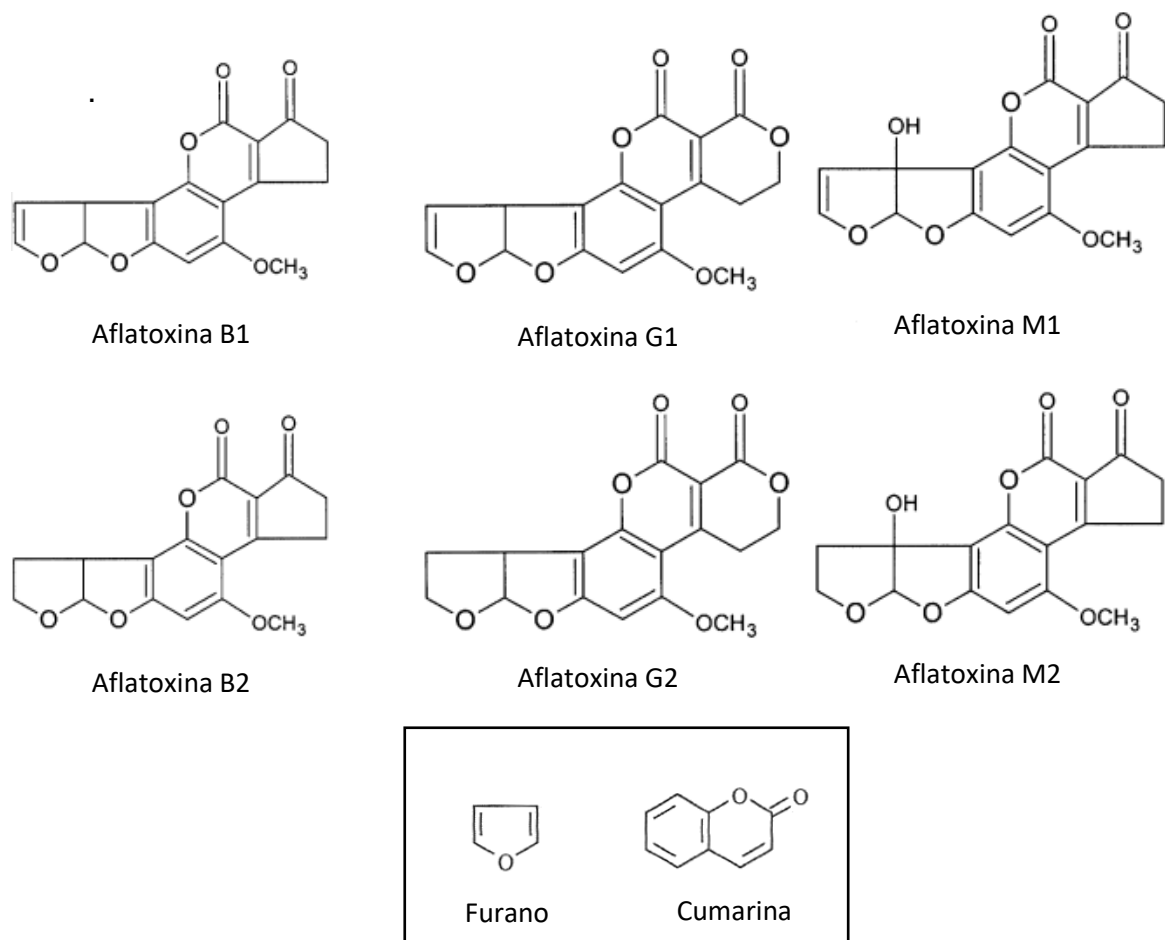


Figura 1 - Estrutura química das principais aflatoxinas e das moléculas de furano e cumarina que fazem parte da estrutura desses compostos tóxicos

As variações que diferem as aflatoxinas estruturalmente estão associadas as suas atividades biológicas. Há cerca de 20 diferentes tipos de aflatoxinas, no entanto entre esses compostos estão as aflatoxinas B₁, B₂, G₁, G₂, M₁ e M₂, que se destacam por seus efeitos tóxicos. Esta toxicidade diminui na seguinte ordem: B₁>M₁>G₁>B₂>M₂=G₂ (BENNETT; KLICH, 2003; SHERIF et al., 2009; HOVE et al., 2016). Além disso, a aflatoxina B₁ (AFB₁) apresenta maior potencial carcinogênico e ocorrência em alimentos, inclusive dentre as micotoxinas. Sua fórmula química é C₁₇H₁₂O₆ e apresenta massa molar de 312,27 g/mol (DHANASEKARAN et al., 2011).

Enquanto as aflatoxinas B₁, B₂, G₁ e G₂ são produzidas naturalmente pelos fungos e estão presentes numa variedade de grãos e rações, as toxinas do tipo M₁ e M₂ são produtos da biotransformação das aflatoxinas B₁ e B₂, respectivamente, que ocorrem no organismo animal após o consumo de ração contaminada, sendo detectada no leite e produtos lácteos (RICHARD, 2007; MARIN et al., 2013; SCAGLIONI et al., 2014; FONTAINE et al., 2015).

As aflatoxinas, assim como outros compostos heterocíclicos, apresentam fluorescência e são distinguidas por esta propriedade. As do tipo B produzem uma fluorescência azul e as do grupo G fluorescem na coloração amarelo-esverdeada sob luz ultravioleta (ROCHA et al., 2014).

Como citado anteriormente, as aflatoxinas são reconhecidas como agentes carcinogênicos para humanos (IARC, 1993, 2012). Além disso, teratogenicidade, imunossupressão, anemia, diminuição da fertilidade e efeito anticoagulante têm sido observados (SHERIF et al., 2009). Efeitos da toxicidade aguda também podem ocorrer e são caracterizados por danos hepáticos, tais como ascite, degeneração hepática, proliferação do ducto biliar e necrose hepática (CULLEN; NEWBERNE, 1994; MCKEAN et al., 2006; NAULT, 2014). De acordo com Rocha et al. (2014), estudos apontam que a presença de aflatoxinas em alimentos é considerada um fator importante no desencadeamento de câncer hepático, uma vez que são rapidamente absorvidas e metabolizadas principalmente no fígado. Foi demonstrado em várias partes do mundo que as aflatoxinas podem contribuir significativamente para aumentar o risco de câncer de fígado em portadores do vírus da hepatite B (BRYDEN, 2012; ROCHA et al., 2014).

A presença de aflatoxinas em alimentos tem sido relatada mundialmente, principalmente em amendoim, nozes, milho, arroz, frutas secas, temperos, cacau, feijão, sorgo, cevada e algodão (BENFORD et al., 2010; HAMMAMI et al., 2014; OK et al., 2014; BARANYI et al., 2015; MASOOD et al., 2015; KABAK, 2016; KAMIKA et al., 2016). Estas micotoxinas são encontradas em alimentos devido à contaminação por fungos durante a pré e pós-colheita. Países tropicais e subtropicais são favoráveis para a produção de aflatoxinas em decorrência do clima quente e úmido (PATERSON; LIMA, 2011; ASHIQ et al., 2014).

No Brasil, as aflatoxinas têm sido encontradas especialmente em amendoim e seus derivados (HOELTZ, 2008; ATAYDE et al., 2012). No entanto, trabalhos também descrevem sua ocorrência em milho e derivados (MORENO et al., 2009; JAGER et al., 2013; SOUZA et al., 2013), castanha do Pará e castanha de caju (FREIRE et al., 2000; MASSI et al., 2014; MILHOME et al., 2014; REIS et al., 2014), trigo (TROMBETE et al. 2014), ração para gado, leite e derivados (PEREIRA et al, 2005; IHA et al., 2011; OLIVEIRA et al., 2013; SCAGLIONI et al., 2014; IQBAL et al., 2015; CAMPAGNOLLO et al., 2016).

1.3 Ocratoxina A

Após a descoberta das aflatoxinas foi dado início a vários estudos extensivos visando identificar novas espécies de fungos e moléculas de micotoxinas. Em 1965, experimentos realizados em ração para aves apontaram que algumas linhagens de um fungo similar ao *A. flavus*, denominado de *A. ochraceus*, produziam metabólitos secundários bastante tóxicos, recebendo o nome de ocratoxinas (VAN DER MERWE et al., 1965; SHIBAMOTO; BJELDANES, 1993; DUARTE et al., 2010).

As ocratoxinas são compostos policetídeos que apresentam derivados de desidroisocumarina com ligação amida a um grupo β -fenilalanina (Figura 2), sendo que entre os derivados dessa estrutura, a ocratoxina A (OTA) é o metabólito marcadamente mais tóxico (EL KHOURY; ATOUI, 2010). Esta micotoxina apresenta a fórmula química $C_{20}H_{18}O_6NCl$ e massa molar de 403,82 g/mol (RINGOT et al., 2006).

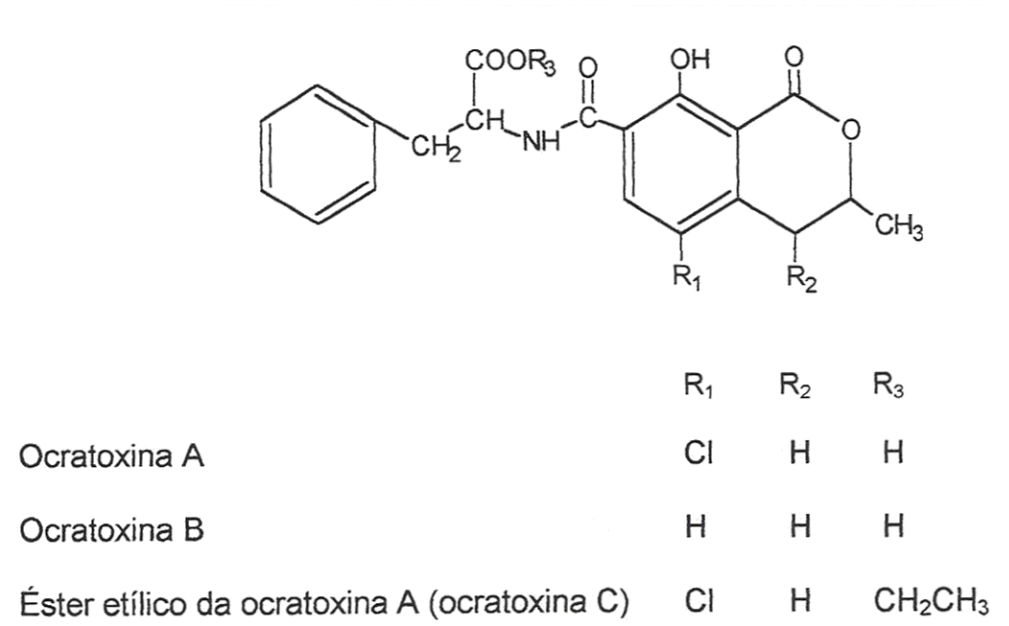


Figura 2 - Estrutura química das principais ocratoxinas

A OTA é produzida por fungos dos gêneros *Aspergillus* e *Penicillium*, o primeiro é bem adaptado a regiões de climas tropicais e subtropicais (crescimento ótimo entre 24 e 37 °C), enquanto o segundo se desenvolve mais em áreas de clima frio (temperatura ótima de 20 °C), podendo ser encontrado em zonas temperadas e frias (RINGOT et al., 2006; PATERSON; LIMA 2011; PATERSON et al., 2014).

Os principais produtores de OTA são *A. ochraceus* e *P. verrucosum*, mas também tem sido relatado constantemente a presença de linhagens de *Aspergillus* da seção *Nigri* (*A. carbonarius* e *A. niger*) produtoras de OTA em países da América do Sul (VALERO, 2008; ASTORECA et al., 2009; KAPETANAKOU et al., 2009; CABAÑES et al., 2010). *Aspergillus melleus*, *A. sulphureus*, *A. cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobulosus*, *A. westerdijkiae*, *A. sulphurous*, *Penicillium viridicatum*, *P. cyclopium*, *P. commune*, *P. purpurecens*, *P. nordicum*, *P. palitans*, *P. thymicola*, entre outros, já foram descritos como produtores de OTA (FRISVAD et al., 2004a; CABAÑES et al., 2010; KHALESÍ; KHATIB, 2011; NGUYEN et al., 2016).

Este micotoxina é considerada nefrotóxica e imunossupressora. Estudos em animais não ruminantes (aves e suínos) apontaram efeitos carcinogênicos, mutagênicos, teratogênicos, genotóxicos e hepatotóxicos (RINGOT et al., 2006; SAVA et al., 2006; ROSSIELLO et al., 2008; VETTORAZZI et al., 2014). Um dos relatos mais conhecidos de sua toxicidade ao homem foi a Nefropatia dos Balcãs, caracterizada por redução progressiva das funções renais. Neste episódio, o surgimento de vários casos de câncer no trato urinário foi relacionado ao consumo de alimentos que continham OTA (DUARTE et al., 2010; VETTORAZZI et al., 2013). Devido a sua carcinogenicidade em animais, a IARC classifica a ocratoxina A no grupo 2B (possível carcinógeno humano).

Ao atingir o fígado a OTA é biotransformada em vários metabólitos que podem estar associados à morte celular por distúrbios na respiração mitocondrial e danos ao DNA e RNA pela formação de adutos (RINGOT et al., 2006). Devido a sua toxicidade, o interesse pela OTA presente nos alimentos, rações e matérias-primas vem aumentando. Sua ocorrência já foi constatada em cereais como milho, trigo, soja, cevada, centeio, arroz, amendoim e café, e ainda em temperos, chocolate, frutas secas, uva, sucos de frutas e bebidas fermentadas, tais como cerveja e vinho (KAWASHIMA et al., 2007; KAPETANAKOU et al., 2009; DUARTE et al., 2010; MAKUN et al., 2013; PATERSON et al., 2014; SARIGIANNIS et al., 2014; KARA et al., 2015; LEE; RYU, 2015). Produtos animais também podem estar contaminados com OTA, incluindo carne suína e ovos, devido ao acúmulo da micotoxina nos tecidos pelo consumo de ração contaminada (DUARTE et al., 2012).

No Brasil, a OTA tem mostrado uma baixa frequência de ocorrência (RODRIGUEZ-AMAYA; SABINO, 2002), aparecendo em até 50% das amostras de milho, trigo, arroz e feijão de vários estados brasileiros (FURLONG et al., 1999; JUAN et al., 2008; DORS et al., 2011; ALMEIDA et al., 2012). Há também relatos de ocorrência em café (TANIWAKI et al., 2014), temperos (SHUNDO et al., 2009), cacau e chocolate (MAGALHÃES et al., 2011; COPETTI et al., 2012). A presença de OTA em vinhos nacionais também tem sido descrita na literatura (ROSA et al., 2004; CHULZE et al., 2006; SHUNDO et al., 2006; WELKE et al., 2010; TEIXEIRA et al., 2011).

1.4 Outras micotoxinas

O termo "micotoxinas emergentes" foi atribuído às toxinas com pouco conhecimento disponível. Estudos estão sendo realizados para determinar a sua ocorrência e os potenciais efeitos destes compostos na saúde (VACLAVIKOVA et al., 2013). Neste grupo incluem micotoxinas como: patulina, alcaloides do ergot, eniantinas, toxinas de *Alternaria*, citreoviridina, moniliformina, beauvericina, ácido ciclopiazônico, roquefortina C, ácido micofenólico, griseofulvina, fusaproliferina, penitrem(s), ácido penicílico, verruculogeno e citrinina (JESTOI, 2008; MALACHOVA et al., 2011; PEREIRA et al., 2014). Esta última vem se destacando devido a sua co-ocorrência e efeito sinérgico com a ocratoxina A (CAST, 2003; BRAGULAT et al., 2008; GAYATHRI et al., 2015).

A citrinina, um composto benzopirano (Figura 3) com fórmula molecular $C_{13}H_{14}O_5$ e massa molar de 250,25 g/mol, foi isolada pela primeira vez em 1931 a partir de metabólitos secundários de *Penicillium citrinum*. Inicialmente descrita como um antibiótico, somente em 1955 foi classificada como micotoxina devido a sua toxicidade ao fígado e rins (WANG et al., 2005; ROCHA et al., 2014). Estudos ainda demonstram efeitos negativos sobre a fertilidade em ratos (LIU et al., 2012; QINGQING et al., 2012).

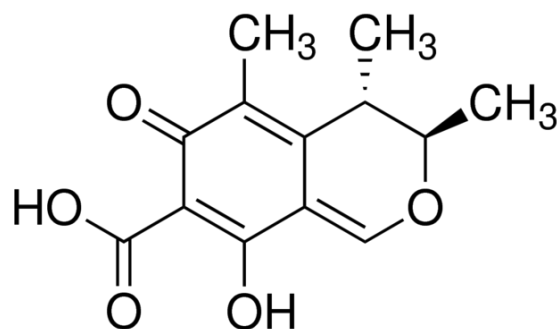


Figura 3 - Estrutura química da citrinina

A citrinina foi associada à síndrome do “arroz amarelo” no Japão em 1971, devido à ocorrência de *P. citrinum* neste produto (SAITO et al., 1971) e também apontada como responsável por causar nefropatia em porcos e outros animais (CARLTON; TUIE, 1977). Além de ser considerada nefrotóxica, é geralmente encontrada em associação com a ocratoxina A (FLAJS; PERAICA, 2009) aumentando a toxicidade.

A contaminação por citrinina foi descrita em arroz, aveia, centeio, cevada, milho, trigo e ração (CAST, 2003; BRAGULAT et al., 2008; ZAIED et al., 2012; LIAO et al., 2014; FERRE, 2016). Apesar de *P. citrinum* ser considerado o principal produtor, *P. expansum*, *P. viridicatum*, *P. gorlenkoanum*, *P. hetheringtonii*, *P. miczynskii*, *P. chrzaszczii*, *P. manginii*, *P. westlingii*, *A. niveus*, *A. terreus* e *Nigrospora oryzae* também são capazes de produzir citrinina, incluindo fungos utilizados na indústria de alimentos como *P. camemberti*, *A. oryzae*, *Monascus ruber* e *M. purpureus* (LIN et al., 2008; JIA et al., 2010; HOUBRAKEN et al., 2011; DONG et al., 2014; LIAO et al., 2015; FERRE, 2016). Curiosamente, *P. verrucosum*, um dos mais importantes produtores de ocratoxina A, também é capaz de sintetizar citrinina (CAST, 2003; BRAGULAT et al., 2008).

1.5 Ocorrência de fungos após o processamento de alimentos

A presença de fungos em alimentos processados pode ser um indicativo de vários fatores, como por exemplo a falta de higiene durante o processo de fabricação ou a utilização de matéria-prima de baixa qualidade, incluindo o uso de embalagens ou ingredientes contaminados (ICMSF, 2005; NDAGIJIMANA et al., 2008). Falhas durante ou após o processamento, como a falta de monitoramento totalmente efetivo das condições ambientais no armazenamento e transporte dos produtos também podem justificar a ocorrência de contaminação fúngica (ICMSF, 2005; GIANNAKOUROS et al., 2005). Além disso, Frisvad e Samson (1991) afirmam que algumas espécies de fungos são adaptadas ao crescimento sob determinadas condições, como baixos valores de atividade de água, temperatura e pH.

A contaminação fúngica pode ocorrer quando boas práticas de fabricação em conjunto com um plano de monitoramento ambiental não são bem aplicados. A falta de cuidados com qualidade do ar de ambientes em que os produtos permanecem após certas etapas do processamento, antes de serem embalados, é um dos exemplos que pode resultar na contaminação superficial de alguns alimentos (GUERRERO; CHABELA, 2000). Kure et al. (2003) consideraram o ar da sala de embalagem de queijos como um ponto crítico para a contaminação do produto por fungos do gênero *Penicillium*. Diversos trabalhos relatam a presença de fungos toxigênicos em queijo (KURE; SKAAR, 2000; MOREIRA et al., 2001; ALY et al., 2010; HYMERY et al., 2014), incluindo *Penicillium roqueforti*, *P. verrucosum*, *P. nordicum*, *P. nalgiovense*, *P. commune* e *P. solitum* (ALY et al., 2010; MIOSO et al., 2014), além de espécies dos gêneros *Aspergillus* e *Fusarium* (BIAŁASIEWICZ; KŁOSIŃSKI, 2001; HYMERY et al., 2014). A maioria destes fungos foi descrita como produtores de OTA, roquefortina C, ácido ciclopiazônico, ácido micofenólico, rugulovasinas, esterigmatocistina e ácido kójico (FILTENBORG et al., 1996; FRISVAD et al., 2004b; ALY et al., 2010; HYMERY et al., 2014).

O emprego de tratamentos térmicos durante o processamento de muitos alimentos é comumente utilizado para tentar reduzir os riscos de multiplicação microbiana (JAY, 2005; RAJKOVIC et al., 2010). Em certos casos, como temperaturas de pasteurização normalmente aplicadas a produtos vegetais (70 – 90 °C), tais medidas podem não ser eficazes para inativar alguns fungos (SANT´ANA et al., 2010). Muitos fungos deteriorantes de frutas podem ser termorresistentes, principalmente alguns representantes dos gêneros *Byssochlamys*, *Talaromyces*, *Neosartorya* e *Thermoascus* (PITT; HOCKING, 2009; HOSOYA et al., 2014). Sua detecção já foi relatada em uma grande variedade de produtos como sucos de maçã (SALOMÃO et al., 2014), abacaxi (FERREIRA et al., 2011; ZIMMERMANN et al., 2011) e laranja (SUGAI et al., 2002), e também em néctares de maracujá e abacaxi (FERREIRA et al., 2011).

As principais espécies de fungos termorresistentes encontradas em alimentos, *Byssochlamys fulva* e *B. nivea*, são produtoras de patulina, ácido bissoclâmico, bissotoxina A, assimetrina e variotina (TOURNAS, 1994; ZIMMERMANN et al., 2011; EVELYN; SILVA, 2015). Yilmaz et al. (2014) comentam que *Talaromyces macrosporus*, *T. flavus*, *T. purpurogenus*, *T. islandicus*, *T. bacillisporus*, *T. helicus*, *T.*

stipitatus, *T. trachyspermum* e *T. wortmannii* são descritos como deteriorantes de sucos de frutas e seus derivados. Além disso, algumas espécies também são capazes de produzir micotoxinas como rubratoxina, cicloclorotina, islanditoxina, dentre outras. De acordo com Evelyn et al. (2016), *Neosartorya fischeri* é outra espécie termorresistente que merece destaque devido à produção de fumitremorginas, verruculogena e terreína.

A inibição do crescimento microbiano em alimentos mais perecíveis geralmente é controlada por refrigeração ou congelamento (JAMES, 2006). No entanto, certas espécies de fungos são capazes de se desenvolver em determinados alimentos mesmo quando armazenados em baixas temperaturas (FRISVAD; SAMSON, 1991). Estudos têm verificado a presença de fungos psicrófilos e psicrotróficos em iogurte, leite fermentado, queijo e até mesmo em produtos cárneos. Ndagijimana et al. (2008) comentam que espécies dos gêneros *Aspergillus*, *Penicillium* e *Fusarium* são frequentes na indústria de laticínios. Espécies de *Geotrichum*, *Rhizopus*, *Mucor* e leveduras também são comumente encontradas como contaminantes desses produtos (NDAGIJIMANA et al. 2008; LEDENBACH; MARSHALL, 2009; GOUGOULI et al., 2011). A germinação de esporos e o crescimento micelial de fungos psicrófilos podem ocorrer na superfície do iogurte, por exemplo, mesmo quando o produto está armazenado em baixas temperaturas e dentro do prazo de validade (GOUGOULI et al., 2011). Além do desenvolvimento fúngico, a síntese de micotoxinas também pode ocorrer em baixas temperaturas. A produção de ácido micofenólico (uma micotoxina imunossupressora) foi verificada em iogurtes após o crescimento de *P. brevicompactum* (NDAGIJIMANA et al., 2008).

A capacidade de crescimento fúngico também tem sido verificada em produtos congelados. Wigmann et al. (2015) observaram que *nuggets* de frango congelados em temperaturas de 0 a -5 °C sofreram deterioração por espécies de *Penicillium*, como *P. polanicum* e outros isolados, capazes de produzir *in vitro* uma micotoxina denominada verrucosidina. Embora a produção deste composto só tenha sido confirmada pelos autores após cultivo a 25 °C, estudos demonstram a capacidade de *P. polanicum* em produzir outras micotoxinas em temperaturas entre 0 e 4 °C, como por exemplo, a síntese de OTA (NORTHOLT et al., 1979; NÚÑEZ et al., 2000).

No caso de alimentos com baixa atividade de água, apesar de não permitirem o crescimento da maioria dos microrganismos (JAY, 2005; SPERBER, 2009), também

podem ser suscetíveis ao desenvolvimento de certos fungos. Estudos demonstraram que espécies xerofílicas de *Aspergillus* e *Penicillium*, além das formas sexuais de *Aspergillus*, tais como *Eurotium* e *Emericella*, são contaminantes frequentes de produtos com baixa atividade de água como chocolate, frutas secas e cristalizadas, doces e compotas, peixes submetidos ao processo de salga e ainda em carnes e linguiças curadas (VYTRÁSOVÁ et al., 2002; CERVENY et al., 2009; GRAM, 2009; THOMPSON, 2009).

Ainda há relatos que alguns fungos xerofílicos em alimentos também podem ser toxigênicos. *P. brevicompactum* é um dos fungos pertencentes a este grupo sendo conhecido por produzir ácido micofenólico (SHEN et al., 1996; NAKAGAWA-YOSHIDA et al., 1997). Al-Sohaibani et al. (2011) isolaram *A. flavus* e *A. niger* de chá preto que, além desses fungos serem xerofílicos, demonstraram potencial em produzir aflatoxina. Da mesma forma, vários representantes fúngicos isolados de produtos cárneos curados têm sido constatados como produtores de AFB1, OTA e patulina (IACUMIN et al., 2009; MARTÍN et al., 2004; RODRÍGUEZ et al., 2012). Copetti et al. (2011) verificaram a presença de *Eurotium amstelodami*, *E. rubrum*, *A. niger*, *A. flavus*, *Paecilomyces variotii* e *Penicillium citrinum* em chocolate em pó, atribuindo como indícios de contaminação no decorrer do processo de fabricação. De Clercq et al. (2015) isolaram espécies de *Penicillium*, principalmente *P. brevicompactum*, e *Eurotium* em produtos de confeitaria, incluindo chocolate. De acordo com os autores, estes organismos podem ser provenientes do ambiente de fábrica ou dos ingredientes utilizados, como por exemplo castanhas e nozes.

Independente do potencial toxigênico dos fungos presentes em alimentos processados, a contaminação fúngica continua sendo de grande preocupação para a indústria alimentar. A ocorrência desses microrganismos em alimentos industrializados também compromete a qualidade dos produtos devido a sua deterioração, causando alterações na textura, aroma e sabor, dentre outras características que afetam a aparência do produto e ainda reduz seu tempo de prateleira (VYTRÁSOVÁ et al., 2002; PITT; HOCKING, 2009; SPERBER, 2009).

De acordo com Van Long et al. (2016), ainda é muito difícil obter um quadro preciso da contaminação real dos produtos alimentares provenientes da indústria, uma vez que esta informação é raramente publicada e referências mais recentes não estão disponíveis. Porém, sabe-se que a deterioração fúngica provoca perdas

econômicas significativas para a indústria e comércio (ANDERSEN; THRANE, 2006; TOURNAS; MEMON, 2009; RAWAT, 2015; ALÍA et al., 2016; SNYDER et al., 2016; VAN LONG; DANTIGNY, 2016). Além disso, ainda há rejeição e desperdício do consumidor, acarretando também no retorno destes agentes ao ambiente devido ao descarte de todo material contaminado (alimento e embalagens) no lixo doméstico.

1.6 Controle de micotoxinas

Várias estratégias têm sido investigadas com a finalidade de evitar ou minimizar a contaminação de micotoxinas na cadeia alimentar envolvendo métodos químicos, físicos e biológicos (SEKIYAMA et al., 2007). No entanto, ainda apresentam algumas desvantagens, como custos elevados ou perdas na qualidade do alimento, ou ainda necessitam de estudos mais aprofundados para garantir sua eficiência. A redução da ocorrência e impacto das micotoxinas requer uma compreensão integrada de biologia, agronomia, ecologia de fungos, métodos de colheita, condições de armazenamento, processamento de alimentos e estratégias de desintoxicação (BRYDEN et al., 2012).

Além disso, o desenvolvimento de materiais a partir de pesquisas nanotecnológicas também pode contribuir na busca de outras soluções para o controle de micotoxinas, devido ao grande potencial e versatilidade desta ciência em expansão (BRANDELLI et al., 2012; CHAUHAN, et al., 2015a; WANG et al., 2016). Uma abordagem sobre as possíveis utilizações de produtos em escalas nanométricas para garantir a segurança de alimentos contra a contaminação fúngica será realizada no item 1.8.

1.6.1 Medidas preventivas

Entre as estratégias para controlar a exposição às micotoxinas, a mais simples e eficaz se baseia na prevenção da contaminação dos grãos e alimentos evitando o crescimento fúngico (DIAZ; SMITH, 2005). Muitas das práticas agrícolas (pré e pós-colheita), empregadas para obter a máxima produção e qualidade de grãos, também

têm reduzido a suscetibilidade à infecção por fungos toxigênicos e contaminação por suas micotoxinas, tais como: uso de grãos mais resistentes à infestações (de insetos e fungos), plantio de cultivares adaptados às regiões, fertilização adequada, diminuição dos ataques de insetos, irrigação quando necessário, colheita imediata após maturidade da cultura, cuidados com transporte, monitoramento da umidade e temperatura de armazenamento, entre outras (CLEVELAND et al., 2003; SANTIN et al., 2005; HOELTZ, 2009). Infelizmente, essas práticas nem sempre são possíveis de serem empregadas, geralmente devido aos obstáculos econômicos, e muitas vezes não são suficientes para prevenir a formação de micotoxinas (KLICH, 2007).

Zain et al. (2011) relatam que a colheita precoce reduz a infecção fúngica em certas plantações diminuindo conseqüentemente a contaminação por micotoxinas, no entanto, esta prática não é realizada entre os agricultores na África, que apesar de cientes, são obrigados a coletar em tempo inadequado devido às condições climáticas e financeiras. Outro exemplo, é o desenvolvimento de cultivares que possuem genes de resistência para fungos ou insetos. Protótipos de cultivos geneticamente modificados contendo genes que reduzem a infecção fúngica foram desenvolvidos (CLEVELAND et al., 2003). Apesar de ser uma estratégia promissora, Munkvold (2003) comenta que poucos cultivares comerciais têm níveis adequados de resistência a fungos toxigênicos e, portanto, mais estudos devem ser realizados.

O uso de compostos químicos também é uma estratégia muito atraente para impedir a produção de micotoxinas (FAO/WHO/UNEP, 1999). Contudo, para ser eficiente, os fungicidas devem ser totalmente letais aos microrganismos em questão, caso contrário, podem estimular a produção de micotoxinas (D'MELLO et al., 1998; MATTHIES et al., 1999). Em uvas, por exemplo, alguns fungicidas podem ser eficazes contra a colonização fúngica e produção de OTA (VARGA; KOZAKIEWICZ, 2006). Entretanto, outros agrotóxicos, como por exemplo, o carbendazim, apesar de reduzir a microbiota, estimula a produção de OTA (MEDINA et al., 2007a). Outro exemplo é o composto azoxistrobina (uma estrobilurina), que resultou na redução de *Fusarium* sp. mas aumentou o nível de contaminação com deoxinivalenol (JENNINGS et al., 2000; SIMPSON et al., 2001). Além disso, há uma necessidade de encontrar meios seguros para o controle fúngico, principalmente por causa da toxicidade dos resíduos de fungicidas sintéticos à saúde humana e ao ambiente (ROMEIRO et al., 2005; KONG et al., 2010).

Muitos óleos essenciais de plantas aromáticas, por exemplo, têm sido testados quanto as suas propriedades antifúngicas (TIAN et al., 2014; JUÁREZ et al., 2015; PEKMEZOVIC et al., 2015; BOUBAKER et al., 2016; ELGUEA-CULEBRAS et al., 2016; SANTAMARINA et al., 2016). No entanto, a maioria dos estudos sobre óleos essenciais reforça a necessidade de realizar testes adicionais, como experimentos em campo e avaliação toxicológica. Apesar da ampla possibilidade de aplicações de óleos essenciais, incluindo na indústria de alimentos, Llana-Ruiz-Cabello et al. (2015) comentam que poucos estudos toxicológicos são realizados. O timol, por exemplo, é um dos compostos presentes em muitos óleos essenciais, no entanto, estudos *in vitro* e *in vivo* demonstraram que essa substância apresenta toxicidade moderada (MARCHESE et al., 2016). Portanto, os aspectos prejudiciais de óleos essenciais e seus componentes devem ser considerados para garantir a segurança de sua utilização.

A aplicação de agentes biológicos como medida preventiva para o controle de fungos toxigênicos tem recebido bastante atenção. A capacidade de certos microrganismos para combater o crescimento fúngico em plantas, envolvendo diferentes modos de ação (ROMEIRO et al., 2005), é vista como uma alternativa promissora para a redução ou eliminação do uso de agroquímicos no controle de fungos fitopatógenos (ARREBOLA et al., 2010; TODOROVA; KOZHUHAROVA, 2010).

O controle biológico é uma ferramenta promissora devido a sua quase inesgotável fonte de matéria-prima (EMMERT; HANDELSMAN, 1999). De acordo com a Academia Nacional de Ciências dos Estados Unidos (do inglês: *National Academy of Sciences*, NAS), o controle biológico compreende a utilização de organismos naturais ou modificados, incluindo seus genes, na tentativa de restringir os efeitos de organismos indesejáveis, e favorecer aqueles que apresentem algum benefício, tais como plantas, insetos e microrganismos (STACEY; KEEN, 1996). O antagonismo pode se dar por competição (espaço e nutriente), parasitismo ou predação, indução de respostas sistêmicas de defesa da planta ou por antibiose direta, também chamada de interação amensal (SHARMA et al., 2009; MALLON et al., 2015).

A utilização de microrganismos como bactérias, leveduras, fungos filamentosos e até mesmo vírus como antagonistas de patógenos minimiza o impacto ambiental causado pelos defensivos agrícolas e pode vir a diminuir o custo da produção agrícola.

Existem muitos microrganismos comprovadamente capazes de inibir patógenos *in vitro* e *in vivo* (SHARMA et al., 2009).

Fungos filamentosos são interessantes para o uso no controle biológico. O emprego de *Trichoderma* spp. e *Chaetomium globosum* como antagonistas revelou uma atividade inibitória intimamente relacionada à secreção excessiva de quitinases e de moléculas voláteis com atividade antifúngica (BHUIYAN et al., 2003; AGGARWAL et al., 2004). No entanto, antifúngicos produzidos por fungos filamentosos são incomuns, com poucos relatos na literatura, a exemplo da molécula eringina, produzida por *Pleurotus eryngii* (WANG; NG, 2004).

A aplicação de linhagens de *A. flavus* e/ou *A. parasiticus* não toxigênicas tem demonstrado diminuições nos níveis de aflatoxinas, excluindo por competição as cepas toxigênicas (DORNER et al., 2003; BAPTISTA et al., 2004; VALERO et al., 2007; ARREBOLA et al., 2010). Foram observadas reduções significativas da contaminação por aflatoxina (70 – 99%) após o uso de linhagens não toxigênicas comerciais (Afla-guard® e AF36®) (ATEHNKENG et al., 2008; DORNER, 2009).

No entanto, alguns aspectos ainda devem ser considerados, uma vez que a biologia de populações de *A. flavus* ainda não é bem compreendida. Há evidências suficientes de recombinação sexual com linhagens toxigênicas de *A. parasiticus* e *A. flavus* no campo. Isso poderia acarretar na quebra de eficácia do uso da linhagem não toxigênica devido à produção de novos fenótipos de *A. flavus* resultando em uma maior diversidade no campo (FISHER; HENK, 2012; RAZZAGHI-ABYANEH et al., 2014). Ainda assim, permanece a preocupação em relação à contaminação de grãos e ração que ocorre durante o armazenamento (DORNER, 2005).

Um grande número de bactérias apresenta potencial antagônico contra vários tipos de fungos por possuírem diferentes modos de ação e um amplo espectro de hospedeiros. Diversos gêneros bacterianos têm sido relatados, entretanto os gêneros *Bacillus*, *Paenibacillus*, *Serratia*, *Pseudomonas* e alguns actinomicetos têm se mostrado os mais viáveis. Cerca de 20 a 40% das espécies de *Bacillus* isoladas de solo apresentam algum tipo de antagonismo contra fungos fitopatogênicos (NIELSEN; SORENSEN, 1997; PALUMBO et al., 2006; WALIYAR et al., 2013).

A diversidade microbiana e suas relações antagônicas surgem como ferramentas importantes para a inibição do crescimento de vários fungos influenciando

na produção de micotoxinas (RYAN et al., 2008). Tem sido relatado que a formação de aflatoxina é inibida por muitos fungos e bactérias específicas, como as ácido-lácticas e *Bacillus* spp. (MUNIMBAZI; BULLERMAN, 1998; TODOROVA; KOZHUHAROVA, 2010). Esta inibição pode resultar de vários fatores, incluindo a concorrência por espaço e nutrientes, e a produção de metabólitos (MUNIMBAZI; BULLERMAN, 1998). Estudos vêm sendo realizados para elucidar as interações entre antagonista-patógeno-hospedeiro com o objetivo de estreitar o entendimento entre a ecologia e os mecanismos de ação que permeiam essas interações (ROMEIRO et al., 2005; RYAN et al., 2008).

Na indústria de alimentos, a adoção de boas práticas de fabricação e o emprego de várias estratégias, como por exemplo tratamentos térmicos, armazenamento a frio, utilização de embalagens adequadas (à vácuo ou com atmosfera modificada) ou ainda a adição de conservantes são frequentemente utilizadas em conjunto para prevenir o crescimento fúngico (JAMES, 2006; SPERBER, 2009; RAJKOVIC et al., 2010, ASEFA et al., 2011). No entanto, como citado anteriormente, incidentes podem ocorrer durante o processo de produção favorecendo a contaminação fúngica, principalmente de espécies resistentes ou tolerantes aos tratamentos empregados (GUERRERO; CHABELA, 2000; ICMSF, 2005; NDAGIJIMANA et al., 2008). Além disso, problemas de armazenamento inadequado também podem resultar na formação de um ambiente adequado para o crescimento de fungos (ICMSF, 2005).

1.6.2 Descontaminação

Embora a melhor forma de se controlar a presença de micotoxinas em alimentos e rações seja evitar a sua formação, muito se tem pesquisado sobre métodos de descontaminação. Para tanto, de acordo com Diaz e Smith (2005), os processos a serem utilizados deveriam atender certos requisitos propostos pela Organização das Nações Unidas para Alimentação e Agricultura (do inglês: *Food and Agriculture Organization of the United Nations*, FAO): destruir, inativar ou remover a micotoxina; não produzir ou deixar resíduos carcinogênicos/mutagênicos nos produtos finais ou em produtos obtidos de animais alimentados com rações descontaminadas; manter o valor nutritivo e a aceitação do alimento; não alterar

significativamente as propriedades tecnológicas importantes do alimento; destruir esporos e micélios fúngicos a fim de evitar a formação de outras micotoxinas sob condições favoráveis e considerar os efeitos no ambiente.

Nem sempre os métodos de descontaminação são apropriados devido a uma série de limitações, tais como sua ineficácia contra algumas micotoxinas, perdas no valor nutricional e propriedades organolépticas dos alimentos, bem como o custo elevado para implementar algumas técnicas (TENIOLA et al., 2005; CAST, 2007).

Métodos convencionais de seleção de grãos contaminados são reconhecidos como processos de descontaminação que podem reduzir quantidades significativas de micotoxinas. A separação de grãos intactos daqueles danificados ou descoloridos (embora a contaminação possa estar em grãos aparentemente livres de fungos visíveis), a remoção de grãos via procedimento eletrônico e ainda a utilização de um processo pneumático através de injeção de ar para retirar os grãos mais leves que estão infectados por fungos, são exemplos de procedimentos empregados para o controle de micotoxinas. Contudo, tais processos geralmente não promovem a descontaminação total de determinados produtos (MORENO; KANG, 1999, ZOVICO et al., 1999; OLSSON et al., 2002; BETHKE et al., 2014; TORRES et al., 2014).

A detoxificação, que é a inativação das toxinas ou conversão em compostos com propriedades menos tóxicas, tem sido extensivamente estudada. Processos como a autoclavagem em alta pressão e temperatura, a torrefação, a utilização de agentes oxidantes, raios gama e ultravioleta, ou solventes podem demonstrar bons resultados na diminuição dos teores de micotoxinas, porém, a maioria apresenta aspectos negativos, incluindo eficácia limitada e até mesmo riscos à saúde (MORENO; KANG, 1999).

A utilização de tratamentos térmicos pode causar a degradação de certas micotoxinas, conseqüentemente reduzindo os níveis destes compostos, mas não elimina completamente. A dimensão da redução dos níveis de micotoxinas depende da estabilidade da toxina, tipo e condições de processamento dos alimentos (MORENO; KANG, 1999). Em uma extensa revisão sobre detoxificação de tricotecenos, He et al. (2010) comentam que deoxinivalenol é estável a 120 °C e parcialmente estável a 210 °C. Estes autores ainda reforçam que, em alguns casos, o tratamento térmico poderia causar alterações nutricionais de determinados produtos.

O emprego de irradiação é um processo ainda controverso. Enquanto pesquisas demonstram sua eficiência, sem causar efeitos adversos nas propriedades organolépticas dos produtos, outras apontam resultados pouco satisfatórios (SAMARJEEWA et al., 1990; AQUINO et al., 2005; BETHKE et al., 2014). A variação do percentual de degradação de micotoxinas em alimentos submetidos à irradiação é bastante ampla, além disso esta técnica possui limitações em condições de baixa umidade (HE et al., 2010). Patel et al. (1989) verificaram que a degradação de aflatoxina por irradiação é mais eficiente quando o teor de água é maior. Isto é explicado pela produção de radicais livres durante a radiólise da água que vão reagir com as micotoxinas (STEPANIK et al., 2007).

Quanto à utilização de agentes químicos, apesar da capacidade de certos solventes orgânicos em remover eficientemente a maioria das micotoxinas, problemas relacionados à exposição aos solventes tóxicos impedem sua aplicabilidade em alimentos (CARÃO et al., 2014). Outros compostos químicos também podem auxiliar no processo de descontaminação de micotoxinas. A amônia gasosa e aquosa tem sido uma forma eficiente e barata de detoxificação das aflatoxinas, convertendo-as em produtos menos tóxicos como a aflatoxina D1 (MOSS, 1998). Porém, esta técnica é ineficiente para grandes lotes de grãos, além de causar alterações nutricionais e organolépticas (PIVA et al., 1995; MORENO; KANG, 1999; HE et al., 2010). A oxidação de algumas micotoxinas, geralmente através do emprego de ozônio, vem sendo bastante investigada. Esta técnica pode causar alterações na estrutura molecular destes compostos e conseqüentemente interfere na sua atividade biológica (TIWARI et al., 2010; CHEN et al., 2014; SUN et al., 2016; TORLAK et al., 2016; WANG et al., 2016a). No entanto, alguns fatores como umidade, pH e temperatura podem interferir no processo de oxidação de determinadas micotoxinas (HE et al., 2010; TIWARI et al., 2010).

Algumas espécies de microrganismos, tais como bactérias ácido-lácticas, *Bacillus subtilis*, *Rhodococcus erythropolis*, e os fungos *Aspergillus niger*, *A. parasiticus*, *Trichoderma viride*, dentre outros, foram relatados com capacidade para degradar AFB1 (TENIOLA et al., 2005; ALBERTS et al., 2006; CAO et al., 2011a). Tal efeito também já foi demonstrado com o cogumelo *Armillariella tabescens* (LIU et al., 1998). No entanto, a maioria destes microrganismos não é oficialmente autorizada pela *Food and Drug Administration* (FDA) e *Association of American Feed Control*

Officials (AAFCO) para a utilização em alimentos ou ração, além de ser necessário estudos mais detalhados sobre sua segurança.

Outro procedimento para o controle de micotoxinas é a utilização de adsorventes a fim de impedir a absorção destas toxinas. Adsorventes são suplementos adicionados aos alimentos, que não são absorvidos no trato gastrointestinal, ligando-se às micotoxinas de modo a transportá-las total ou parcialmente para fora do trato digestivo, evitando que sejam absorvidas pelo organismo, impedindo que causem intoxicação (ZHU et al., 2016). Estes agentes englobam desde o carvão ativado, a parede celular de leveduras (*Saccharomyces cerevisiae*) e até mesmo as argilas (minerais silicatos), que constituem um grupo imenso e complexo com vários representantes, como os aluminossilicatos (HSCAS) (DAKOVIKĆ et al., 2005; DIAZ; SMITH, 2005). No entanto, há uma série de fatores que podem interferir na eficiência desta ferramenta (HUWIG et al., 2001).

A diversidade de micotoxinas existentes, variando em suas estruturas químicas e propriedades físicas, impede a atuação dos adsorventes com a mesma eficácia para todas. Os aluminossilicatos naturais, por exemplo, possuem superfícies hidrofílicas e por isso são mais efetivos na adsorção de aflatoxinas e menos eficientes para micotoxinas apolares, como a zearalenona (DAKOVIKĆ et al., 2005) ou ainda podem prejudicar a absorção de algum elemento da dieta (DEVEGOWDA et al., 1998; FRANCISCATO et al., 2006). Além disso, quando chegam ao intestino, os adsorventes são expostos a fatores como pH, temperatura, enzimas e microrganismos, que podem afetar sua capacidade de adsorção.

Carboidratos da parede celular de algumas espécies de leveduras também são outros compostos muito utilizados como adsorventes (HUWIG et al., 2001), porém, trabalhos complementares são necessários para explorar seu potencial (TENIOLA et al., 2005; SEKIYAMA et al., 2007). Curiosamente, Zinedine et al. (2005), demonstraram a capacidade de algumas linhagens de *Lactobacillus rhamnosus* em eliminar a presença de AFB1 em meio de cultura (caldo MRS), todavia por ser uma suposição do modo de ação em um modelo extremamente abstrato, a eficiência real deve ser testada *in vivo*.

1.7 Utilização de bactérias para o controle de fungos e micotoxinas

Os métodos biológicos para o controle de micotoxinas podem incluir a prevenção do crescimento de fungos, seja por competição ou produção de compostos antifúngicos (PALUMBO et al., 2006; DORNER, 2008; PITT; HOCKING, 2009; REDDY et al., 2009), ou ainda podem abranger processos de biodesintoxicação das toxinas (adsorção, biotransformação ou biodegradação). Apesar do número crescente de microrganismos utilizados como estratégias para o controle das micotoxinas, bactérias dos gêneros *Bacillus* e *Pseudomonas* são de maior importância.

1.7.1 O gênero *Bacillus*

Os microrganismos que pertencem a este gênero incluem bactérias geralmente Gram positivas, com forma de bastonetes, aeróbias, raramente anaeróbias facultativas. Este grupo de bactérias se destaca por incluir uma variedade de espécies industrialmente importantes devido a certas características como alta taxa de crescimento, capacidade de secretar proteínas para um meio extracelular, reconhecimento do status GRAS (do inglês: *Generally Recognized as Safe*) de algumas espécies e uma notável capacidade de formar endósporos (SCHALLMEY et al., 2004).

Espécies de *Bacillus* são ubíquas na natureza, porém apesar de terem como habitat natural o solo, já foram isoladas de diversos ambientes como água doce, sedimentos marinhos, água salgada, plantas, animais (incluindo o trato gastrointestinal), ar e poeira, entre outros (GEBHARDT et al., 2002; PIGNATELLI et al., 2009). Estes ambientes fornecem condições ótimas para o crescimento e proliferação deste gênero, assim como a manutenção da sua forma de resistência. A capacidade de distribuição e persistência dos esporos faz com que estas bactérias se mantenham latentes nos mais diversos ambientes, possibilitando dessa forma, a sua longa manutenção e sobrevivência em nichos ecológicos (ORTEGA-MORALES et al., 2009).

A amplitude metabólica de *Bacillus* tem sido aproveitada pela indústria para a produção de moléculas, tais como enzimas, antibióticos, aminoácidos e toxinas de insetos e nematoides (MAUGHAN; VAN DER AUWERA, 2011). O potencial desse gênero em secretar vários peptídeos com capacidade de inibição do crescimento fúngico já é conhecido sendo, portanto, usado amplamente como agente de controle biológico (CHEN et al., 2009; ZHAO et al., 2010; PALAZZINI et al., 2016; ZOUARI et al., 2016).

Os lipopeptídeos provavelmente representam a classe mais comum de metabólitos secundários produzidos por *Bacillus* spp. (STACHELHAUS et al., 2002). Estes compostos são peptídeos não-ribossômicos e estão classificados em três famílias, dependendo da sequência de aminoácidos: surfactinas, iturinas e fengicinas (STEIN, 2005; ONGENA; JACQUES, 2008; ONGENA et al., 2009).

Iturinas e fengicinas apresentam forte atividade antifúngica contra um grande número de fungos fitopatógenos (MOYNE et al., 2004; ROMERO et al., 2007; ARREBOLA et al., 2010; YÁNEZ-MENDIZÁBAL et al., 2011), danificando a membrana celular. Estudos sobre o mecanismo de ação da iturina A demonstram a indução da abertura de canais iônicos nas membranas celulares de fungos e leveduras, causando um desequilíbrio eletroquímico entre os meios intra e extracelular. A surfactina, apesar de não inibir o crescimento do fungo, aumenta a atividade antifúngica da iturina A, através de uma relação sinérgica (COUTTE et al., 2010). Estes compostos, além de apresentarem amplo espectro antifúngico e baixa toxicidade, são facilmente biodegradáveis, tornando-os agentes promissores para o controle biológico (MONTESINOS, 2003; SZCZECH et al., 2006; MIZUMOTO et al., 2007; YE et al., 2012).

Outros compostos antifúngicos produzidos por linhagens de *Bacillus* são bacteriocinas de síntese ribossômica (ZHENG; SLAVIK, 1999; OSCÁRIZ; PISABARRO, 2000), enzimas que degradam a parede celular (tais como proteases, quitinase, β -1,3-glucanase) e algumas proteínas inibitórias não identificadas (MANJULA et al., 2004).

Diversas espécies desse gênero têm sido descritas como antagônicas de fungos fitopatógenos devido à produção destas substâncias, incluindo *B. subtilis*, *B. pumilus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis* e *B. megaterium*

(AHERN et al., 2003; MENDO et al., 2004; ARREBOLA et al., 2010; TODOROVA; KOZHUHAROVA, 2010; PRETORIUS et al., 2015). O envolvimento de lipopeptídeos produzidos por *Bacillus* spp. na inibição do crescimento de fungos toxigênicos, tem sido relatado em vários estudos (KLICH et al., 1993; MOYNE et al., 2001; ZHANG et al., 2008; CHO et al., 2009; PALAZZINI et al., 2016; QIAN et al., 2016). Uma linhagem de *B. megaterium* isolada de ambiente marinho na China reduziu significativamente o crescimento de *A. flavus* e biossíntese de aflatoxinas (KONG et al., 2010). *Bacillus subtilis* pode inibir o crescimento de fungos toxigênicos, incluindo algumas espécies de *Aspergillus* (FÖLDES et al., 2000), tais como *A. flavus* (GONG et al., 2014) e *A. carbonarius*, e *Penicillium*, como por exemplo, *P. expansum* (SENTHIL et al., 2011) e até mesmo controlando o crescimento de *Fusarium verticillioides*, um potencial produtor de fumonisina (CAVAGLIERI et al., 2005).

Além disso, existem relatos de muitas espécies de *Bacillus* capazes de promover o crescimento e resistência de plantas (WANG et al., 2009; WHITE et al., 2014). Bactérias promotoras de crescimento de plantas também secretam frequentemente compostos antimicrobianos e hormônios de crescimento vegetal (AHEMAD; KIBRET, 2014). As espécies de *Bacillus* também são referidas como bactérias endofíticas em plantas superiores, termo utilizado para microrganismos que habitam o interior de plantas (GUPTA et al., 2000; ONGENA et al., 2005; LI et al., 2012; WHITE et al., 2014).

A utilização de *Bacillus* não se limita apenas ao controle biológico, sua aplicação na redução dos níveis de micotoxinas por degradação ou biotransformação desses compostos também vem sendo investigada. Enzimas extracelulares produzidas por *B. subtilis*, *B. pumilus*, *B. megaterium*, *B. licheniformis*, *B. lentus*, *B. firmus* e *B. clausii* foram capazes de degradar aflatoxinas, OTA e zearalenona (CENCI et al., 2008; FUCHS et al., 2008; PETCHKONGKAEW et al., 2008; CHO et al., 2010; TINYIRO et al., 2011; MA et al., 2012).

Algumas linhagens de *Bacillus* provenientes de ambientes aquáticos de Bacia Amazônica, mais especificamente do intestino de peixes típicos da região, vêm sendo extensivamente estudadas quanto ao seu potencial biotecnológico (GIONGO et al., 2007; DAROIT et al., 2009, 2011; CORREA et al., 2010; LEMES et al., 2016). Vários estudos demonstraram a capacidade dessas bactérias em produzir lipopeptídeos com atividade antimicrobiana contra bactérias (CLADERA-OLIVERA et al., 2004; MOTTA

et al., 2004; BRANDELLI et al., 2007, 2014), vírus (SILVA et al., 2014) e fungos não toxigênicos (VELHO et al., 2011). No entanto, o conhecimento sobre o potencial antifúngico desses isolados ainda é limitado, uma vez que até o momento, não houve estudos sobre o controle de fungos toxigênicos e produção de micotoxinas.

1.7.2 O gênero *Pseudomonas*

As espécies do gênero *Pseudomonas* são bastonetes Gram negativos e estritamente aeróbias. A maioria é quase exclusivamente saprófita, habitando solos úmidos, água ou superfície de vegetais. Algumas vivem em estado comensal no intestino dos animais e homem (MICHEL-BRIDAN; BAYSSE, 2002). Isolados bacterianos desse gênero vêm sendo utilizados no controle biológico de fungos e têm recebido atenção pelo fato de apresentarem um rápido crescimento *in vitro* e capacidade de produção de compostos antimicrobianos (HASS; KEEL, 2003).

Vários metabólitos secundários produzidos por *Pseudomonas* são citados como importantes na atividade de controle biológico. Enzimas degradativas, como a protease, celulase, quitinase e β -glucanase em conjunto com antibióticos, tais como fenazinas, floroglucinois, pirrolnitrina e pioluteorina, são os principais compostos produzidos por este gênero. Sideróforos e antifúngicos peptídicos com propriedades surfactantes, como a viscosinamida, além de compostos voláteis, como cianeto de hidrogênio, também podem ser sintetizados por estas bactérias (NIELSEN et al., 1999; HASS; KEEL, 2003, HAAS; DÉFAGO, 2005; WANG et al., 2011; CHEN et al., 2015).

Pseudomonas fluorescens, *P. aeruginosa*, *P. aureofaciens*, *P. putida* e *P. pyrrocinia* são espécies que apresentam atividade antifúngica (GORANTLA et al., 2014). Em relação ao controle de fungos produtores de micotoxinas as duas primeiras são as mais investigadas. Wang et al. (1999a) isolaram de *P. aeruginosa* um carboidrato complexo de alto peso molecular, denominado pafungina, que demonstrou atividade contra *Fusarium oxysporum* (potencial produtor de fumonisina). Enquanto um isolado de *Pseudomonas fluorescens* reduziu em 93% o crescimento de *A. flavus* (REDDY et al., 2009). Além disso, *P. fluorescens* também é reconhecida como agente

indutor de resistência de plantas contra alguns fungos devido à produção de enzimas ou precursores (TROTEL-AZIZ et al., 2008).

Ainda há casos descritos na literatura sobre a degradação de micotoxinas por esse gênero. El-Deeb (2005) relatou que *Pseudomonas* spp. foram capazes de remover zearalenona, embora o produto não tenha sido identificado, foi considerado menos tóxico. O sobrenadante livre de células obtido de dois isolados de *P. putida* causou uma redução máxima de 25,7% na concentração de OTA (RODRIGUEZ et al., 2011). Reddy et al. (2009) reduziram em 83,7% os níveis de AFB1 utilizando um extrato obtido do cultivo de *P. fluorescens*. Recentemente, *Pseudomonas* sp. também foi descrita na degradação de aflatoxina (ELAASER; EL KASSAS, 2011; KRIFATON et al., 2011). Samuel et al. (2014) demonstraram que, em meio de cultivo MSG (glicose e sal mineral), *P. putida* foi capaz de degradar AFB1 em outros compostos menos tóxicos (AFD1, AFD2 e AFD3).

Fontoura et al. (2009) isolaram uma bactéria do gênero *Pseudomonas* a partir da água de tratamento de efluentes de um frigorífico da região do vale do Rio Pardo, Rio Grande do Sul. Este isolado revelou uma ampla atividade antimicrobiana através da produção de uma substância com efeito sobre microrganismos patógenos e também em deteriorantes de alimentos, porém nenhum teste de atividade antifúngica foi realizado. Desta forma, ainda há muitos aspectos para explorar sobre o potencial biotecnológico desta bactéria.

1.8 A nanotecnologia como ferramenta para o controle de fungos

Como citado anteriormente, o crescimento de fungos em alimentos pode ocorrer mesmo após o seu processamento. Desta forma, pesquisas utilizando ferramentas nanotecnológicas também devem ser investigadas para melhorar a qualidade e segurança dos produtos.

A nanotecnologia consiste na capacidade de desenvolver, manipular e caracterizar estruturas e materiais em escala nanométrica, geralmente com tamanho inferior a 100 nm (BRANDELLI, 2012; ADAMS; BARBANTE, 2013; BRYS et al., 2016). A maioria dos materiais em nanoescala possui propriedades significativamente

diferentes daquelas encontradas no mesmo material em tamanho maior, permitindo novas funcionalidades e conseqüentemente grandes avanços científicos (SUH et al., 2009).

Devido às inúmeras possibilidades de aplicações, a nanotecnologia tem atraído a atenção de pesquisadores principalmente nas áreas da física, biologia, química e engenharias (YANG; TIGHE, 2013; SAFARI; ZARNEGAR, 2014; HUSSEIN, 2015; CAYUELA et al., 2016). De acordo com Dong et al. (2016), agências governamentais e indústrias por todo o mundo têm investido milhões de dólares em financiamento de pesquisas nesse domínio, tornando os Estados Unidos e a China líderes globais em nanotecnologias. O Brasil ocupa a décima oitava posição no ranking de pesquisas sobre nanotecnologia. Tal ocupação merece destaque por ser o único representante da América do Sul entre os 20 principais países que mais publicaram artigos relacionados com a nanotecnologia entre o período de 2003 a 2013. Atualmente cerca de 50 universidades e centros de pesquisa nacionais, em colaboração com empresas privadas, estão envolvidos em estudos nesse novo ramo da ciência (com mais de 9 mil publicações científicas) (FONSECA; PEREIRA, 2014; DONG et al., 2016).

A nanotecnologia também tem potencial para desempenhar um papel importante na indústria alimentar, podendo ser empregada para a melhoria de vários atributos dos alimentos e até mesmo contribuir na sua segurança contra a contaminação microbiana (CHAUDHRY et al., 2008). Neste caso, o controle de microrganismos patogênicos e seus compostos produzidos vem sendo investigado, por exemplo, através de estudos utilizando nanosensores e nanosondas, os quais identificam quando o produto está contaminado, evitando assim o consumo (DUNCAN, 2011). A maioria dos trabalhos está relacionada com a detecção de bactérias e vírus, havendo poucos relatos sobre a utilização de nanosensores para fungos (HEO; HUA, 2009; TALLURY et al., 2010; COMINI et al., 2013; SACHDEV et al., 2016). Estudos recentes desenvolveram imunossensores utilizando nanopartículas de ouro e prata para a detecção de micotoxinas (CHAUHAN et al., 2015a, b; PAGKALI et al., 2017; WANG et al., 2016b), porém a eficiência desses sensores em diferentes produtos alimentares ainda deve ser verificada. Além disso, os nanosensores desenvolvidos eram específicos apenas para AFB1, AFM1 ou OTA.

Outra abordagem nanotecnológica para o controle de microrganismos em alimentos se dá pela investigação de materiais com propriedades antimicrobianas. Nanopartículas de ouro, prata, cobre e platina, ou ainda outros nanomateriais inorgânicos como óxidos de zinco, cálcio e magnésio ou dióxido de titânio apresentam atividade antimicrobiana (HUANG et al., 2005; EGGER et al., 2009; MARY et al., 2009; SÁNCHEZ-VALDES et al., 2009; HAMAL et al., 2010; AZAM et al., 2012;; RHIM et al., 2013). Pesquisas têm demonstrado que a utilização dessas partículas em materiais poliméricos, geralmente para a fabricação de embalagens, poderia complementar as estratégias de controle microbiano. Pinto et al. (2013) testaram a atividade antifúngica de filmes polissacarídicos de pululana incorporados com nanopartículas de prata contra *A. niger*. Maneerat e Hayata (2006) avaliaram o efeito *in vitro* de uma película plástica de polipropileno revestida com nanopartículas de dióxido de titânio contra *P. expansum*, confirmando influência do material sobre o crescimento do fungo. Os resultados foram semelhantes ao aplicar a película funcionalizada em limões e tomates. Nanopartículas de óxido de zinco foram testadas contra *Botrytis cinerea* (KAIRYTE et al., 2013) e *P. expansum* (HE et al., 2011), mas nestes estudos, as nanopartículas não foram revestidas com quaisquer filmes.

Apesar dos efeitos benéficos que as nanopartículas podem exercer, devido à ação antimicrobiana ou detecção de contaminação nos alimentos, o conhecimento sobre suas implicações na saúde humana e no ambiente ainda é limitado (SILVESTRE et al., 2011). A exposição às nanopartículas pode ocorrer através do contato com a pele, inalação ou ingestão de partículas que migraram para o alimento (WANG et al., 2007; CARLSON et al., 2008; LI; HUANG, 2008; RESTUCCIA et al., 2010). Alguns estudos têm sugerido que esses nanomateriais podem apresentar efeitos toxicológicos nos sistemas biológicos (DONALDSON; SEATON, 2007; OBERDÖRSTER et al., 2007). Além disso, o desenvolvimento de nanopartículas resulta no aumento de emissões para o meio ambiente, porém o impacto ambiental desses materiais depende de suas propriedades físicas e químicas, além das características do local onde serão depositadas (VAN LONG et al., 2016). Alguns trabalhos ainda comentam que as nanopartículas podem se acumular nos seres vivos e entrar indiretamente na cadeia alimentar (HOET et al., 2004; SILVESTRE et al., 2011).

1.8.1. Nanofibras

A obtenção de fibras poliméricas com dimensões em escalas nanométricas (10 – 1000 nm de diâmetro) é outro exemplo de nanoestruturas com grande potencial de uso em importantes campos da pesquisa e tecnologia. Dentre os procedimentos de fabricação de nanofibras, a técnica de eletrospiação (do inglês: *electrospinning*), na qual utiliza forças eletrostáticas, tem recebido enorme atenção devido à sua versatilidade para produzir fibras ultrafinas a partir de uma variedade de materiais poliméricos (HUANG et al., 2003; CHEN; CHANG, 2011; ZULKIFLI et al., 2014). Além disso, é uma técnica relativamente simples e de baixo custo quando comparada com outros métodos de fabricação (CELEBIOGLU et al., 2014).

No processo de *electrospinning*, uma solução do polímero, armazenada em uma seringa com agulha metálica, é submetida a um campo eletrostático através de uma fonte de alta tensão que possui dois eletrodos de cargas elétricas opostas. Um eletrodo da fonte é colocado na agulha da seringa e o outro, conectado a um coletor de metal (KE et al., 2014). Quando uma certa voltagem é aplicada, de modo que ultrapasse a tensão superficial da gota polimérica presente na extremidade da agulha, ocorre deformação dessa gota, originando um filamento que será ejetado em direção ao coletor (INGAVLE; LEACH, 2014). À medida que o jato polimérico percorre esse trajeto, o solvente utilizado na solução evapora permitindo a solidificação e deposição do material formado (PHAM et al., 2006). Um esquema da produção de nanofibras pela técnica de *electrospinning* pode ser melhor visualizado na Figura 4.

De acordo com Brandelli (2012), o processo de eletrospiação tem permitido o desenvolvimento de nanofibras a partir de vários polímeros sintéticos ou naturais, como por exemplo quitina, acetato de quitosana, ácido poli-L-lático, álcool polivinílico e poli- ϵ -caprolactona (PCL). Este último é um polímero sintético linear, hidrofóbico, e assim como os demais poliésteres alifáticos possui baixa toxicidade e biodegradabilidade, sendo aplicado a uma variedade de produtos aprovados pela FDA (GAUTAN et al., 2013; JI et al., 2014; SIAFAKA et al., 2016).

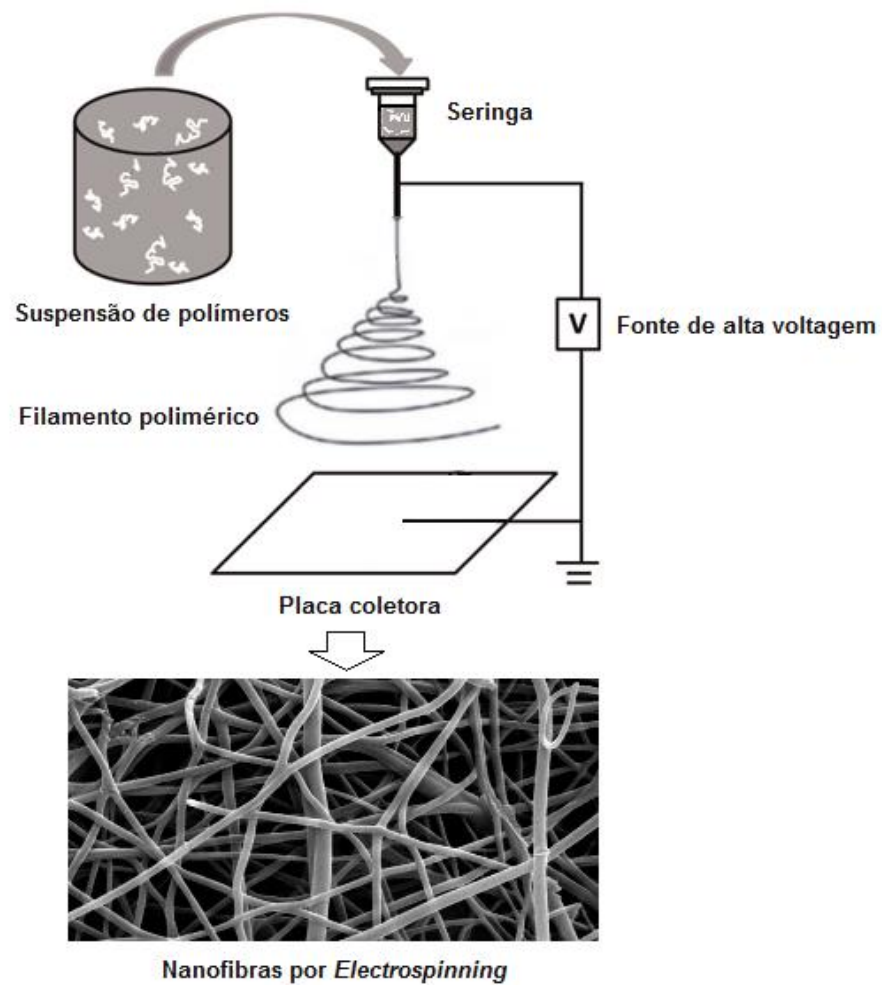


Figura 4. Representação esquemática da produção de nanofibras através da técnica de *electrospinning*, adaptado de Zhong et al. (2015)

As nanofibras obtidas por *electrospinning* também podem ter suas propriedades modificadas alterando os parâmetros do processo (ZULKIFLI et al., 2014). Isso favorece o surgimento de características interessantes como aumento da área de superfície por volume e a possibilidade de incorporação de substâncias específicas, permitindo determinada funcionalização química devido à posterior liberação controlada do composto incorporado (CANBOLAT et al., 2014). Ao considerar essas propriedades, as nanofibras “eletrofiadas” vêm sendo reconhecidas como promissoras na área de alimentos para diversas aplicações, tais como produção de filmes comestíveis (quando se tratar de polímeros naturais) ou ainda na elaboração de embalagens que permitam a liberação controlada de agentes antimicrobianos, flavorizantes, antioxidantes, sequestrantes de oxigênio, absorventes de umidade, sensores ou outros componentes bioativos incorporados no interior ou superfície

dessas estruturas (BHUSHANI; ANANDHARAMAKRISHNAN, 2014; BLANCO-PADILLA et al., 2014; KHAN; OH, 2016; RAJ; SHANKARAN, 2016).

Em relação à atividade antimicrobiana de nanofibras funcionalizadas, vários agentes antimicrobianos têm sido incorporados nessas nanoestruturas durante a técnica de eletrofiação, com posterior confirmação de sua atividade devido à liberação do composto após determinado período de tempo (CELEBIOGLU et al., 2014). Como por exemplo em nanofibras de derivados de celulose e álcool polivinílico que demonstraram atividade antibacteriana contra *Escherichia coli* e *Staphylococcus aureus* (JIA et al., 2011). Heunis et al. (2010), observaram atividade antimicrobiana de nanofibras de polietileno incorporadas com plantaricina 423 (uma bacteriocina), sendo eficaz na inibição do crescimento de *Enterococcus faecium* e *Lactobacillus sakei*. Em outro estudo, Nitanan et al. (2013) desenvolveram nanofibras de uma mistura polimérica (poliestireno sulfonado e ácido polivinílico) com neomicina como agente antibacteriano. Finalmente, nanofibras de PCL e óxido de polietileno encapsuladas com lisozima foram capazes de liberar tal enzima após 12 dias (KIM et al., 2007).

Apesar da crescente pesquisa de nanoestruturas funcionalizadas com agentes antimicrobianos, o número de estudos sobre a incorporação de compostos antifúngicos em nanofibras ainda é limitado (Tabela 2). A maioria dos trabalhos refere-se ao desenvolvimento de fibras nanométricas contendo nanopartículas de metais, principalmente prata e cobre (MUÑOZ-BONILLA; FERNÁNDEZ-GARCÍA, 2012; DOBRZAŃSKI et al., 2014; PRAMANIK et al., 2014; IFUKU et al., 2015; SHI et al., 2015). Como exemplo, Quirós et al. (2016) avaliaram nanofibras à base de acetato de celulose com nanopartículas de prata e cobre na inibição de *A. niger*.

Dentre os trabalhos sobre nanofibras funcionalizadas com compostos antifúngicos, destaca-se a pesquisa de Lakshminarayanan et al. (2014) dedicada ao desenvolvimento de nanofibras de gelatina contendo cinco antifúngicos diferentes, incluindo natamicina, nas quais apresentaram atividade contra *C. albicans*, *F. solani*, *A. fumigatus* e *A. brasiliensis*. No entanto, considerando a ampla necessidade de controlar as diversas situações de ocorrência fúngica (infecções e contaminações), é necessário avaliar a utilização de outras matrizes poliméricas para a produção de diferentes tipos de nanofibras e a eficácia dos antifúngicos incorporados.

Tabela 2. Nanofibras funcionalizadas com compostos antifúngicos obtidas através da técnica de *electrospinning*

Matriz polimérica	Composto	Fungo	Referência
PVA/óxido de polietileno	Metronidazol	<i>Aspergillus flavus</i> , <i>A. niger</i> , <i>Penicillium notatum</i>	El-Newehy et al. (2012)
Quitosana/EDTA/PVA	Clotrimazol	<i>Candida albicans</i>	Tonglairoum et al. (2015a)
Quitosana/PVP /PVA	Clotrimazol	<i>C. albicans</i>	Tonglairoum et al. (2015b)
PVP/hidroxipropil- β -ciclodextrina	Clotrimazol	<i>C. albicans</i>	Tonglairoum et al. (2014)
Polianilina	Fluconazol	<i>C. albicans</i> , <i>C. tropicalis</i> e <i>C. krusei</i>	Yehgambaram et al. (2013)
Zeína	Ácido gálico	<i>C. albicans</i>	Neo et al. (2013)
Poliacrilonitrila	Amidoxima	<i>Saccharomyces cerevisiae</i>	Sirelkhatim et al. (2015)
Poliuretano	Itraconazol	Não testado	Verreck et al. (2003)
PVP	Indometacina e Griseofulvina	Não testado	Lopez et al. (2014)
Fibroína de seda/ácido hialurônico	Extrato de folha de oliveira	<i>C. albicans</i>	Doğan et al (2016)
Nylon	Corantes Xantenos	<i>A. fumigatus</i>	Kim e Michielsen (2015)
Acetato de celulose	Saponina	<i>P. roqueforti</i> e <i>A. ochraceus</i> ,	Dixit et al. (2010)
Gelatina	Anfotericina B, Natamicina, Cloridrato de terbinafina, Itraconazol, Fluconazol	<i>C. albicans</i> , <i>F. solani</i> , <i>A. fumigatus</i> e <i>A. brasiliensis</i>	Lakshminarayanan et al. (2014)
PVA	Fluconazol	<i>C. albicans</i>	Sharma et al. (2014)
PCL/óxido de polietileno	Clotrimazol	<i>C. albicans</i>	Mir et al. (2014)

PVA (Álcool polivinílico), EDTA (ácido etilenodiamino tetra-acético), PVP (polivinilpirrolidona), PCL (policaprolactona)

Vale ressaltar que até o momento não há relatos de nanofibras incorporadas com cetocoazol (independente da matriz polimérica). Este antifúngico, pertencente à classe dos imidazóis, é bastante reconhecido pelo seu amplo espectro de ação interferindo na síntese de ergosterol da membrana fúngica (STAUB et al., 2005; RANE; PADMAJA, 2012). O desenvolvimento de nanofibras de PCL funcionalizadas com cetoconazol, além de ser bastante atrativo para a utilização em tratamentos clínicos de infecções fúngicas (caso haja liberação controlada), também pode servir como modelo para pesquisas sobre a incorporação de outros compostos antifúngicos. Isto poderia possibilitar a utilização de nanofibras como material antimicrobiano em inúmeras aplicações, incluindo na indústria de alimentos.

A natamicina, por exemplo, é outro composto de grande relevância para estudos sobre sua incorporação em nanofibras. Produzido principalmente pela bactéria *Streptomyces natalensis*, este antifúngico do grupo dos macrolídeos polienos interfere na permeabilidade da membrana celular devido à formação de canais iônicos após sua ligação com as moléculas de esterol da mesma (PASEIRO-CERRATO et al., 2013). A maioria dos fungos contaminantes de alimentos é suscetível à ação da natamicina, além disso também é relatado seu potencial de inibir ou reduzir a síntese de algumas micotoxinas como AFB1, OTA, patulina e ácido penicílico (DELVES-BROUGHTON et al., 2005; MEDINA et al., 2007b).

Sua utilização como conservante de alimentos é recomendada e a maioria dos países possui legislações que permitem o uso apenas para alguns alimentos, principalmente queijos e produtos cárneos embutidos (EUROPEAN UNION, 1995; BRASIL, 1988, 2001; JECFA, 2002). No entanto, países como China e África do Sul ainda estendem sua utilização para outros tipos de alimentos, como produtos de panificação, iogurte, sucos de fruta e vinhos.

De acordo com Reys et al. (2002) e Fucinos et al. (2012), a aplicação de natamicina sobre superfícies de alimentos, por pulverização ou imersão, nem sempre é tão eficaz, provavelmente devido a sua hidrofobicidade. Por isso, outras estratégias para a aplicação em alimentos vêm sendo extensivamente estudadas, incluindo sua adição em filmes poliméricos (KOONTZ; MARCY, 2003; TÜRE et al., 2008; BALAGUER et al., 2014; VAN LONG et al., 2016). A incorporação de natamicina em nanofibras de PCL, a caracterização dessas estruturas funcionalizadas e seu efeito *in vitro* contra fungos ainda não foram estudados.

CAPÍTULO 2

Inhibition of mycotoxin-producing fungi by *Bacillus* strains isolated from fish intestines

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Abstract

Bacillus strains isolated from aquatic environment of Brazilian Amazon region were tested for their activity against mycotoxigenic fungi. All bacteria showed antifungal activity, inhibiting at least 7 indicator fungi. Four *Bacillus* strains showing promising antifungal results were subsequently evaluated for their activity in reducing mycelial growth rate, sporulation, spore germination percentage, and mycotoxins production. *Bacillus* sp. P1 and *Bacillus* sp. P11 had a remarkable antifungal effect on toxigenic fungi. Washed bacterial cell suspension of strains P1 and P11 (10^7 CFU/ml) reduced by more than 70% the fungal colony diameters, including a complete inhibition of *Aspergillus* sp. producer of ochratoxin A (OTA). Significant reduction of growth rate, sporulation and spore germination were also observed. The bacteria influenced the production of mycotoxins, causing a reduction around 99 and 97% in AFB1 and OTA concentration, respectively. Chromatographic analysis revealed the presence of lipopeptides (iturin A and surfactin isomers) in butanol extracts of cell-free supernatants and cell pellets of strains P1 and P11. Furthermore, antifungal activity of these extracts was confirmed against *A. flavus* and *A. carbonarius*, producers of AFB1 and OTA, respectively. These bacterial strains could be promising biocontrol agents against toxigenic fungi.

Keywords: *Bacillus* species; biocontrol; iturin; mycotoxins; surfactin

1. Introduction

Mycotoxins are harmful substances whose occurrence in food, even at low concentrations but consumed for long periods, can result serious injury to human and animal health (Proietti et al., 2014). They represent a group of toxic substances that display varied chemical, physical and toxicological properties, but share the fact that they are secondary metabolites from biosynthetic routes of certain fungi (Pitt and Hocking, 2009, Marin et al., 2013; Ashiq et al., 2014).

Mycotoxin contamination of foodstuffs and agricultural commodities has been reported worldwide. Climatic conditions, associated with inadequate agricultural practices, may determine the occurrence of fungal species and the type of mycotoxins produced (Kumar et al., 2008; Pitt and Hocking, 2009). Species of genera *Aspergillus*, *Penicillium* and *Fusarium* are among the most important toxigenic fungi and are frequently reported in the literature due to the occurrence in foods and potential for mycotoxin production (Barrett, 2000; Simas et al., 2007).

Among all the relevant mycotoxins in food, special mention is made to aflatoxin B1 (AFB1) and ochratoxin A (OTA) because of their high toxicity as well as for their carcinogenic, mutagenic and teratogenic properties (Marin et al., 2013; Ashiq et al. 2014; De Ruyck et al., 2015). The International Agency for Research on Cancer (IARC) assessed the carcinogenic potential of these compounds and classified aflatoxins, including AFB1, as carcinogens to humans (Group 1), whereas OTA is categorized into group 2B, a possible carcinogenic agent to humans (IARC, 1993 and 2012). Furthermore, some mycotoxins should receive more attention because of their co-occurrence with other ones, as is the case of citrinin, frequently associated with OTA (CAST, 2003; Marin et al., 2013). These toxins may act synergistically increasing the probability of toxic effects on an organism (Gayathri et al., 2015).

In addition to health problems, significant economic losses are associated with mycotoxin contamination at all levels of the food chain. Many strategies have been used to prevent mycotoxicosis. Chemical, physical and biological methods have been investigated to reduce or eliminate mycotoxins from different foods. However, such methods are frequently limited by economic reasons, partial efficiency, or alteration of food quality parameters (Jouany, 2007; Klich, 2007). Therefore, the prevention of mycotoxin synthesis remains best way to avoid food contamination. In this case, use of synthetic fungicides may be an attractive strategy to control fungal growth. However,

these agents must be completely lethal, otherwise they may stimulate the production of mycotoxins (D'Mello et al., 1998; Matthies et al., 1999), or constant use can result in the development of fungal resistance (Krid et al., 2012). Moreover, there is a need to find safe means of controlling these pathogens due to the impact of fungicides on human health and the environment.

The inhibition of toxigenic fungi by antagonistic microorganisms arise as an interesting alternative to control mycotoxin contamination in food. Among the most promising candidates to be used as biological control agents, the genus *Bacillus* has been intensively investigated due to certain characteristics such as high growth rate, recognition of GRAS status (Generally Recognized as Safe) of some species, ability to form endospores, and production of a large number of antimicrobial substances (Schallmeyer et al., 2004). The potential of *Bacillus* species to produce lipopeptides with ability to inhibit fungal growth is already known (Chen et al., 2009; Zhao et al., 2010). These compounds are non-ribosomal peptides that have low toxicity, high biodegradability and are classified into three major families: iturins, fengycins and surfactins (Ongena and Jacques, 2008; Caldeira et al., 2011). Iturins and fengycins have strong antifungal activity against a large number of phytopathogenic fungi (Romero et al., 2007a; Arrebola et al., 2010; Yáñez-Mendizábal et al., 2011). Although surfactins are not antifungal substances, they have a synergistic action with iturins and fengycins, increasing their antifungal activity (Coutte et al., 2010).

In recent years, some *Bacillus* strains from the aquatic environment of the Amazon region were investigated for their antimicrobial activity against bacteria (Cladera-Olivera et al., 2004; Motta et al., 2004), viruses (Silva et al., 2014) and non-toxigenic fungi (Velho et al., 2011). However, the knowledge about the antifungal potential of these isolates is still limited, since no studies were conducted on the control of toxigenic fungi and mycotoxin production. The aim of this study was to evaluate the ability of *Bacillus* strains isolated from fish intestines to affect the growth parameters (mycelial growth rate, sporulation and spore germination percentage) of toxigenic fungi *in vitro* and the mycotoxins production. The ability of these bacterial strains in production of lipopeptides iturin A and surfactins was also investigated.

2. Materials and methods

2.1. Microorganisms and maintenance

Bacillus strains, isolated from intestines of typical fishes of the Brazilian Amazon region (Table 1), were obtained from the culture collection of the Laboratório de Bioquímica e Microbiologia Aplicada (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). Some isolates were selected based on their inhibitory activity against phytopathogenic fungi (Velho et al., 2011). *B. subtilis* ATCC 19659 (American Type Culture Collection, Rockville, USA) was also included in the study as a control.

Table 1

Bacterial strains tested for inhibition of toxigenic fungi.

Bacteria isolates	Origin
<i>Bacillus subtilis</i> ATCC 19659	American Type Culture Collection
<i>Bacillus</i> sp. P1	<i>Potamorhina latior</i>
<i>Bacillus</i> sp. P7 ^a	<i>Piaractus mesopotamicus</i>
<i>Bacillus</i> sp. P11 ^a	<i>Leporinus</i> sp.
<i>Bacillus</i> sp. P34 ^a	<i>Leporinus</i> sp.
<i>Bacillus</i> sp. P39A	<i>Leporinus</i> sp.
<i>Bacillus licheniformis</i> P40 ^a	<i>Leporinus</i> sp.
<i>Bacillus subtilis</i> P45B ^a	<i>Piaractus mesopotamicus</i>
<i>Bacillus</i> sp. P51	<i>Semaprochilodus</i> sp.
<i>Bacillus</i> sp. B312	<i>Myletus edulis</i>

^a Bacteria tested by Velho et al. (2011) against phytopathogenic fungi.

The following toxigenic isolates of fungi were used in this investigation: *Aspergillus flavus* A12, *Aspergillus* sp. A65 and *A. parasiticus* 30BL (aflatoxin B1 producers); *A. carbonarius*, *Aspergillus* sp. UMO1B, *Aspergillus* sp. UCO1A,

Aspergillus sp. UCO2A and *Aspergillus* sp. UCS2B (ochratoxin A producers); *Penicillium citrinum* and *Monascus purpureus* NRRL1992 (citrinin producers); *P. chrysogenum* IFL1 and *P. chrysogenum* IFL2 (roquefortine C producers) and *Fusarium graminearum* (fusarenon X producer). All fungi are kept in the culture collection of the Laboratório de Toxicologia de Alimentos (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). The strains *A. carbonarius* and *P. citrinum* were kindly provided by Instituto de Tecnologia de Alimentos (ITAL, Campinas, Brazil).

The bacterial cultures were stored on Brain Heart Infusion (BHI; Oxoid, Basingstoke, UK) agar plates at 4°C or for long term storage at -20°C in BHI broth with 20% (v/v) glycerol and routinely propagated twice from frozen stocks before use. The fungi were maintained on potato dextrose agar (PDA; Acumedia, Lansing, MI, USA) slants covered with mineral oil at 4°C and subcultured periodically. Working isolates were cultivated onto PDA agar plates.

2.2. Inocula production

Inoculations of bacterial cells were made from the working cultures into BHI broth and incubated at 37°C for 24 h with constant shaking (125 rpm). The concentration of cell suspension was determined by spectrophotometry (optical density at 600 nm) and viability confirmed by standard plate count method using BHI agar. The concentration of each bacterial suspension was adjusted to obtain approximately 10^7 CFU/ml.

To prepare fungal inoculum, each isolate was grown on PDA plates at 25°C for 7 days and 12 h photoperiod. Spores were harvested from plates by adding 5 ml of sterile distilled water containing 0.05% (v/v) Tween 80 and with the aid of a Drigalski loop. The suspension was collected and transferred to a sterile tube. Then, the material was filtered through sterile gauze to ensure spores free of any debris. Finally, spores were counted with a Neubauer chamber and the suspensions were used in the experiments.

2.3. Initial selection of potentially antagonistic bacteria

Bacterial isolates were screened for antagonism *in vitro* against the toxigenic fungi. The method described by Benitez et al. (2010) was followed with minor modification. Fifteen milliliters of sterile PDA at 45°C containing fungal spore suspension (1×10^6

spores/ml) were transferred to Petri dishes and allowed to solidify. The PDA plates were surface inoculated with 20 μ l of *Bacillus* liquid culture (10^7 CFU/ml), cultured previously on BHI broth at 37°C and 125 rpm during 48 h.

The Petri dishes were incubated at 25°C for 5 days and then, the areas of inhibition were measured with a digital caliper. The antagonistic activity was evaluated according to the scale proposed by Bacon and Hinton (2002): -, no antagonism; +, weak antagonism (clear zones of inhibition < 3 mm); ++, moderate antagonism (clear zones of inhibition \geq 3 - 9 mm); +++, strong antagonism (clear zones of inhibition > 9 mm). The bacteria with more representative results were subjected to further studies.

2.4. Bacterial effect on fungal growth

The most promising four bacterial strains were further tested to determine the effects on fungal growth, colony characteristics and sporulation of two representative producers of AFB₁, OTA or citrinin. This evaluation was adapted from Nesci et al. (2005) and Gandomi et al. (2009). Bacteria cells were obtained by centrifugation at 10,000 rpm for 15 min after growing as previously described. Each bacterial suspension (10^7 CFU/ml) was mixed with 15 ml of molten PDA medium and poured into Petri plates (80 mm diameter). After the plates were cooled, a sterile paper disc (5 mm diameter) was placed at the center of agar surface and 10 μ l of fungal spore suspension (10^6 spores/ml) were spotted onto the discs. The control plates were inoculated with test fungi but without bacteria. *B. subtilis* ATCC 19659 was also tested following the same procedure. All plates were subsequently incubated at 25°C for 10 days. The diameters of fungal colonies were measured daily in two directions at right angles to each other during this time and used to plot growth curves. The absence of mycelial mass in the surface of plate was considered as 100% inhibition.

Percentage inhibition of mycelial growth was determined by comparing the growth in the plates containing both microorganisms with the control cultures and calculated according the following equation:

$$\text{Inhibition of growth (\%)} = ((D_c - D_t) / D_c) \times 100 \quad (1)$$

where D_c represents the diameter of fungal colony in control plate and D_t the diameter of fungal colony in treated plate containing the antagonist bacterium.

When the hyphae in the control plate reached the maximum diameter of the plate (80 mm) or after 10 days, the colony growth rate (mm/day) of each plate was determined by linear regression of the linear phase for growth.

After growth was evaluated, all samples were analyzed for bacterial influence on fungal spore production. The spores produced were washed by 15 ml Tween 80 solution (0.05%) and the estimate of conidial concentration (spores/cm² of colony) was performed using a Neubauer chamber. The following formula was employed in order to calculate the percent of sporulation inhibition:

$$\text{Inhibition of sporulation (\%)} = ((N_c - N_t) / N_c) \times 100 \quad (2)$$

where N_c represents the number of fungal spores produced in control plate and N_t is the number of spores produced in treated plate containing the antagonist bacterium.

2.5. Spore germination inhibition assay

Bacillus strains were tested for their effect on conidia using a modified procedure of Wang et al. (2008) and Gameda et al. (2014). Fungal spore suspensions (10⁶ spores/ml) were prepared as described earlier and 100 µl of each suspension was added into sterile glass tubes containing 800 µl of YES broth (2% yeast extract and 20% sucrose; pH 6.5) plus 100 µl of *Bacillus* cells (10⁷ CFU/ml). Control tubes received sterile saline solution (0.85%) instead of bacterial cells and used to comparison. All tubes were incubated at 25°C for 24 h and then conidia germination was evaluated by using a light microscope. Spores were regarded to have germinated when the length of germination tube was equal or longer than the diameter of the conidium. Four counts of 100 conidia per repetition were performed and the percent spore germination determined by dividing the number of germinated spores over the total number of spores counted. Percent spore germination inhibition was determined as follows:

$$\text{Inhibition of spore germination (\%)} = ((G_c - G_t) / G_c) \times 100 \quad (3)$$

where G_c represents the percentage of spore germinated in the control tube and G_t is the percentage of spore germinated in treated tube containing the test bacterium.

2.6. Effect of *Bacillus* on mycotoxin production

Bacteria exhibiting strongest antagonistic activity were tested for influence in mycotoxin production. In this case, the best producers of AFB1 and OTA were selected as target fungi. PDA plates containing bacterial cells and test fungi were prepared as previously described (section 2.4). After 10 days incubation, the mycotoxins were extracted as described by Nesci et al. (2005) with some modifications. A piece of agar medium (1 x 1 cm), including microbial biomass, was taken from the fungal colony of each treatment, transferred to a 2 ml Eppendorf tube and 500 µl of chloroform was added. The mixture was shaken at 100 rpm for 30 min and then the chloroform extract was poured into a glass vial. This procedure was repeated twice and the chloroform extracts were combined, filtered through PTFE 0.22 µm syringe filter (Millipore, USA), transferred to a new vial and dried under nitrogen gas. Afterwards, the residue was redissolved in 250 µl of the mobile phase as necessary for high performance liquid chromatography (HPLC) analysis.

Mycotoxins determination was performed using an HPLC instrument (Waters, model E2695) equipped with a fluorescence detector (FL-2475). The separation, identification and quantification steps of AFB1 and OTA were carried out according to Xu et al. (2013) and the AOAC 2001.01 methods (AOAC, 2001), respectively. Separations were performed on a C18 column (XBridge™ Shield RP 18; 5 µm; 4.6 x 150 mm, Waters, Ireland) connected to a precolumn (XBridge™ Shield RP 18; 5 µm; 4.6 x 20 mm, Waters). All analyses were carried out under isocratic conditions using a mobile phase of acetonitrile:water:acetic acid (99:99:2, v/v/v) at a flow rate of 1.0 ml/min and the column oven temperature set to 30°C. The injection volume of sample on column was 20 µl. The fluorescence detector was operated at 360 nm for excitation and 440 nm for emission on AFB1 analysis. Regarding OTA detection, the excitation and emission wavelengths were set to 333 and 460 nm, respectively.

Standard curves were constructed under these conditions with different levels of each mycotoxin. The retention time of AFB1 and OTA was 3.9 and 8 min, respectively. Quantification of these toxins was performed by correlating peak area of sample extracts and those of standard curves. Average recovery values, from PDA agar added with mycotoxins, were 98.9 ± 1.0 and $99.0 \pm 1.2\%$ for AFB1 and OTA, respectively.

2.7. Extraction and analysis of lipopeptides

The best two bacterial isolates from all the experiments and *B. subtilis* ATCC 19659 were tested for the ability to produce lipopeptides, more specifically iturin and surfactin. Bacterial cells were cultivated in 250 ml Erlenmeyer flasks containing 50 ml of BHI medium at 37°C with continuous shaking (125 rpm) for 48 h. After growth, bacterial cells were removed from the broth by centrifugation at 10,000 rpm for 15 min at 4°C in order to separate the supernatant and the cell pellet, which were subjected to extraction of extra and intracellular lipopeptides, respectively.

Extracellular lipopeptides extraction was carried out by addition of *n*-butanol (1:4, v/v) into tubes containing the supernatant. The tubes were vortexed for 1 min and maintained overnight at room temperature under static conditions. The organic phase was collected and the solvent evaporated from the extract at 30 °C in an oven (Yazgan et al., 2001). The material was dissolved in 2 ml methanol and filtered before quantification by HPLC.

For lipopeptides extraction from the cells, the pellet obtained was washed with ultrapure water and centrifuged under the same conditions as above. This procedure was repeated twice. The material was suspended in methanol, homogenized and incubated overnight at 4°C. After that, the extract was centrifuged again and the organic phase was collected followed by filtration prior to analysis.

The obtained lipopeptides were analyzed and quantified by HPLC. For iturin determination (Yuan et al., 2011), a 20 µl sample was eluted isocratically at a flow rate 0.6 ml/min using 0.1% acetic acid:acetonitrile (60:40, v/v) with column oven temperature set to 20°C. A photodiode array detector (PAD-2998) was employed and iturin peaks were detected at 230 nm. Surfactin separation was done following the method proposed by Liu et al. (2012) with minor modifications. Briefly, the mobile phase employed was methanol:trifluoroacetic acid (0.1%) (90:10, v/v). Samples (40 µl) were eluted isocratically at a flow rate 0.3 ml/min and column temperature was adjusted to 20°C. Surfactin peaks were detected at 210 nm. These conditions allowed elution of three surfactin isoforms (C-13, C-14 and C-15).

Iturin A (>90%) and surfactin (>98%) powder were purchased from Sigma Chemical (USA) and each compound was dissolved with 1 ml methanol. Different concentrations from these solutions were prepared (20, 40, 60, 80, 100, 120 and 140 mg/l) in order to develop a calibration curve using the same chromatographic

conditions described above. The retention time of iturin A and surfactin isomers (C-13, C-14 and C-15) was 8, 10.5, 12 and 13 min, respectively. Linear regression equations were obtained and quantification of these lipopeptides was performed by correlating peak area of sample extracts and standard curves.

2.8. Statistical analysis

All data were expressed as the means \pm SD (standard deviation) of three replicates. The results were subjected to analysis of variance (ANOVA) using SAS for Windows ver. 9.0 (SAS Institute Inc., Cary, NC). Differences were considered to be significant at a 95% confidence level by using the Tukey Test.

3. Results

3.1. Antagonistic activity

Bacillus spp. were screened for their effectiveness to control toxigenic fungi on PDA medium. All bacterial strains showed an apparent antagonistic effect against at least 50% of the fungal isolates tested. Among the bacteria tested, *Bacillus* sp. strains P1, P7, P11, P45 and B312 showed the best results, since they inhibited a large number of fungi with inhibition zones ranging from 3 to 9 mm (Table 2). Moreover, other bacteria tested had variable antifungal capability, as the strain P29A that showed a broad inhibitory spectrum but showed large inhibitory zones against a single isolate. The strain *B. subtilis* ATCC 19659, used as negative control in this test, did not show any antagonism.

The lower activity was found against *Aspergillus* strains that are aflatoxin B1 producers. None of the bacteria tested produced inhibitory zones against *A. flavus* A12, although there was no fungal growth on the spots where the strains P1, P7 and P11 were inoculated. *Aspergillus* sp. UCO2A (ochratoxin A producer) was considered the most sensitive fungus since 7 *Bacillus* strains formed inhibition halos greater than 10 mm. Curiously, an inhibition of spore formation was observed around some bacterial colonies on plates containing *A. carbonarius*.

Based on these results, strains P1, P7, P11 and B312 were selected for further investigation. Two fungal isolates producers of AFB₁, OTA and citrinin were used as test organisms in the following assays.

Table 2

Antagonistic activity of *Bacillus* spp. against toxigenic fungi cultivated on PDA plates at 25°C for 5 days.

Fungal isolates	Antagonism of <i>Bacillus</i> strains ^a									
	ATCC	P1	P7	P11	P39A	P34	P40	P45	P51	B312
<i>A. flavus</i> A12	-	- ^b	- ^b	- ^b	-	-	-	-	-	-
<i>Aspergillus</i> sp. A65	-	-	+	++	+	+	++	+	-	-
<i>A. parasiticus</i> 30BL	-	+	++	++	++	-	-	+	-	++
<i>A. carbonarius</i>	-	++	++	++	+	-	++	++	++	++
<i>Aspergillus</i> sp.UMO1B	-	++	++	++	+	-	++	++	++	++
<i>Aspergillus</i> sp. UCS2B	-	++	++	++	+	-	++	++	++	++
<i>Aspergillus</i> sp.UCO2A	-	+++	+++	+++	-	++	+++	+++	+++	+++
<i>Aspergillus</i> sp. UCO1A	-	++	++	++	+	-	++	++	++	-
<i>P. citrinum</i>	-	++	++	++	+	++	-	++	-	++
<i>Monascus purpureus</i>	-	++	++	++	+	++	-	++	+	++
<i>P. chrysogenum</i> IFL1	-	++	++	+	+	+	+	++	++	++
<i>P. chrysogenum</i> IFL2	-	++	++	++	+	++	+	++	++	++
<i>F. graminearum</i>	-	+	-	++	-	++	-	-	+	++
Number of inhibited isolates	0	11	11	12	10	7	8	11	9	10
Moderate or strong antagonism	0	9	10	11	1	5	6	9	7	10

^a -, no antagonism; +, weak antagonism (clear zones of inhibition < 3 mm); ++, moderate antagonism (clear zones of inhibition ≥ 3 - 9 mm); +++, strong antagonism (clear zones of inhibition > 9 mm).

^b Fungal growth was not superimposed to bacteria.

3.2. Growth and sporulation inhibition test

Four pre-screened antagonistic *Bacillus* were tested against six toxigenic fungi in order to observe their effects on fungal growth parameters. The results are summarized in Figure 1 and Table 3. These strains significantly reduced ($P<0.05$) the mycelial growth compared to the diameter of the fungal colonies cultivated simultaneously with *B. subtilis* ATCC 19659, which showed similar values to that observed in control plates (without any bacteria). All bacterial strains were effective for reducing mycelial growth at levels above 60%. In particular, *Bacillus* sp. P11 showed the best results on inhibition of aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) decreasing mycelial growth by 84.2 and 90.5%, respectively. Besides, there was no development of *Aspergillus* sp. UCO2A isolate therefore, it was considered a 100% reduction in growth. *Bacillus* sp. P1 also caused a significant growth reduction of the same fungus (Figure 1 and Table 3).

In general, OTA producers fungi were more sensitive to inhibition by bacterial strains. Beyond *Aspergillus* sp. UCO2A, which was not able to grow when cultivated with *Bacillus* sp. P1 or *Bacillus* sp. P11, the isolate *A. carbonarius* treated with washed cell suspension of *Bacillus* exhibited colonies much smaller than control ($P<0.05$), with diameters ranging from 7.8 to 13.4 mm. Both of fungi grown without bacteria and with *B. subtilis* ATCC 19659, achieved colony diameter of 80 mm (Table 3).

About the producers of citrinin, mycelial growth was considerably reduced with the presence of bacterial cells. However, *B. subtilis* ATCC 19659 also caused a decrease in *M. purpureus* growth, although the final value of colony diameter was significantly different ($P<0.05$) when compared with the other *Bacillus* strains and control treatments.

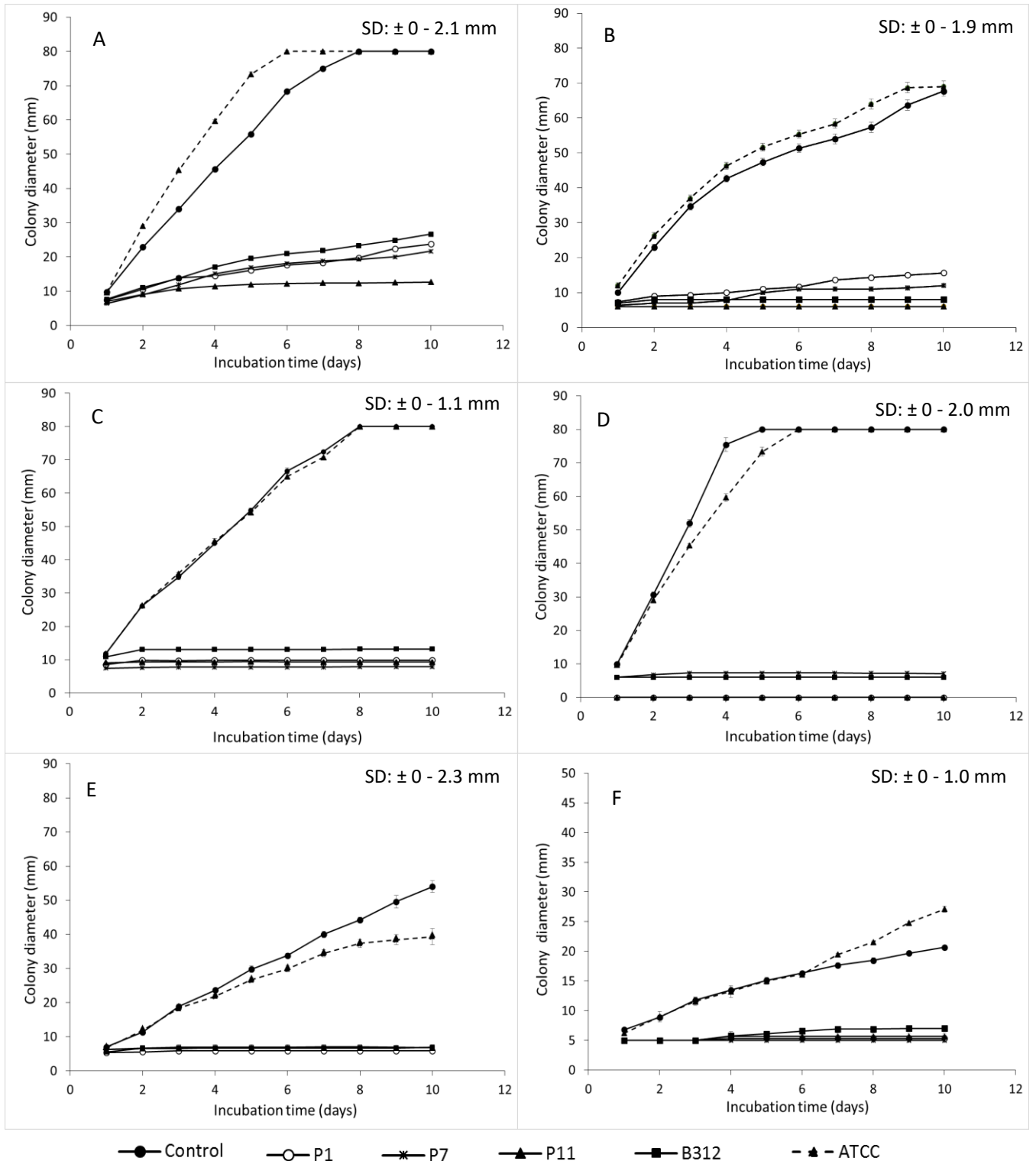


Fig. 1. Profile of mycelial growth of *Aspergillus flavus* (A), *Aspergillus parasiticus* (B), *Aspergillus carbonarius* (C), *Aspergillus* UCO2A (D), *Monascus purpureus* (E) and *Penicillium citrinum* (F) co-cultured with different isolates of *Bacillus* spp. (10^7 CFU/ml) and without bacteria (control) after 10 days of incubation at 25°C on PDA plates, measured as colony radius (mm). The abbreviation SD refers to the variation of the standard deviation.

Table 3

Effect of *Bacillus* spp. on mycelial growth of toxigenic fungi incubated at 25°C for 10 days, measured as percentage of colony diameter reduction for fungi treated with bacteria and diameter values of fungal colony (mm) for treatments of isolates without bacteria (control).

Fungal isolates	Mycelial growth reduction (%)					Control colony diameter (mm)
	<i>Bacillus</i> strains					
	P1	P7	P11	B312	ATCC	
<i>A. flavus</i> A12	70.2±1.39 ^{b,c}	72.9±0.92 ^b	84.2±0.58 ^a	66.7±2.21 ^c	0±0.00 ^d	80.0±0.00
<i>A. parasiticus</i> 30BL	77.2±0.31 ^d	82.0±0.18 ^c	90.5±0.69 ^a	86.5±1.35 ^b	0±0.00 ^e	66.8±0.63
<i>A. carbonarius</i>	87.6±0.52 ^b	90.1±0.23 ^a	88.3±0.00 ^b	83.2±0.35 ^c	0±0.00 ^d	80.0±0.00
<i>Aspergillus</i> sp. UCO2A	100±0.00 ^a	91.1±0.51 ^c	100±0.00 ^a	92.5±0.00 ^b	0±0.00 ^d	80.0±0.00
<i>Monascus purpureus</i>	89.1±0.57 ^a	87.4±0.88 ^a	87.4±0.88 ^a	87.2±0.97 ^a	27.2±1.69 ^{b*}	53.9±1.31
<i>Penicillium citrinum</i>	74.1±2.33 ^a	75.7±0.33 ^a	72.5±2.61 ^a	66.2±1.85 ^b	0±0.00 ^c	20.6±0.26

^{a, b, c} The same letters in each line indicate no significant differences among the data at 5% level by Tukey test. Values are the mean ± standard deviation: average of two experiments carried out in three replicates. *Values of colony diameter are significantly different by the Tukey test when compared with the control (without bacteria).

The values of colony growth rate were calculated (Table 4), and confirmed the previous results. Most fungi co-inoculated with *Bacillus* spp. had a very slow growth and a constant speed, with rate values below 1.0 mm/day. This decrease in growth rates were significant ($P < 0.05$) with respect to the control (fungal isolates cultivated without bacteria), which exhibited values above 5.2 mm/day, except for *P. citrinum* (1.5 mm/day). *Bacillus* sp. P11 had a great influence on growth rate reduction; for example, decrease the growth rate of *A. flavus* from 10.3 to 0.7 mm/day. Furthermore, *B. subtilis* ATCC 19659 shown a stimulatory effect on *P. citrinum* from the seventh day of cultivation, once mycelial growth was faster than the control condition (2.23 mm/day) (Figure 1).

After the cultivation period, the number of fungal spores produced by each isolate was determined. As can be seen in Table 5, the bacterial isolates also exerted great influence on sporulation. The best results were obtained by *Bacillus* sp. P11, which significantly ($P < 0.05$) reduced the sporulation of *A. carbonarius*, *A. flavus* and *M. purpureus* by 80.8, 87.6 and 97.2%, respectively. Moreover, this strain completely inhibited (100%) the spore formation by *A. parasiticus* and *P. citrinum*, which showed only the development of hyphae. It was not possible to observe asexual reproduction structures (Supplementary Figure S1).

It was also found that inhibition of fungal growth may not be associated with sporulation reduction (Table 5). Some bacteria that caused reduction of mycelial growth, such as *Bacillus* sp. P7, have a stimulatory effect on sporulation of some fungal strains. The spore number of *A. parasiticus* increased from 4.8×10^5 to 5.5×10^7 spore/cm² when co-cultivated with *Bacillus* sp. P7, while for *Aspergillus* sp. UCO2A the increase was from 8.3×10^4 to 2.5×10^5 spore/cm². The same pattern was observed for *A. parasiticus* simultaneously cultivated with *Bacillus* sp. P1 (5.5×10^7 spore/cm²).

Table 4

Mycelial growth rate of toxigenic fungi co-cultured with different isolates of *Bacillus* spp. and without bacteria (control) after 10 days of incubation at 25°C on PDA plates.

Fungal isolates	Colony growth rate (mm/day)					
	<i>Bacillus</i> strains					
	P1	P7	P11	B312	ATCC	Control
<i>A. flavus</i> A12	1.65±0.05e	1.91±0.12d	0.70±0.01f	2.23±0.05c	9.65±0.04b	10.30±0.05a
<i>A. parasiticus</i> 30BL	0.93±0.01c	0.68±0.01d	0±0.00e	0.05±0.0e	5.88±0.06a	5.72±0.09b
<i>A. carbonarius</i>	0.12±0.02b	0.04±0.03b	0.02±0.01b	0.19±0.04b	9.46±0.03a	8.47±0.05a
<i>Aspergillus</i> sp. UCO2A	0±0.00c	0.32±0.09c	0±0.00c	0±0.00c	15.52±0.10b	18.48±0.13a
<i>Monascus purpureus</i>	0.03±0.04c	0.04±0.01c	0.04±0.00c	0.07±0.02c	3.72±0.22b	5.27±0.15a
<i>Penicillium citrinum</i>	0.04±0.06d	0±0.00d	0.08±0.06d	0.27±0.02c	2.23±0.04a	1.50±0.06b

^{a,b,c} The same letters in each line indicate no significant differences among the data at 5% level by Tukey test. Values are the mean ± standard deviation: average of two experiments carried out in three replicates.

Table 5

Effect of *Bacillus* spp. on sporulation of toxigenic fungi incubated at 25°C for 10 days, measured as percentage of sporulation reduction for fungi treated with bacteria and number of fungal spores for treatments of isolates without bacteria (control).

Fungal isolates	Sporulation inhibition (%)					Control (spores x 10 ⁵ /cm ²)
	<i>Bacillus</i> strains					
	P1	P7	P11	BL47	ATCC	
<i>A. flavus</i> A12	75.8±0.64a	78.1±0.76a	87.6±0.31a	85.6±0.55a	0±0.00b*	1.82±4.14
<i>A. parasiticus</i> 30BL	0±0.00b	0±0.00b	100±0.00a	100±0.00a	0±0.00b	4.82±0.63
<i>A. carbonarius</i>	20.8±1.37c	96.1±0.38a	80.8±1.63a,b	59.1±0.17b	0±0.00c	0.86±0.02
<i>Aspergillus</i> sp. UCO2A	-	0±0.00b	-	57.7±1.22a	4.9±2.76b*	0.83±0.21
<i>Monascus purpureus</i>	76.2±0.11a	96.7±0.85a	97.2±2.17a	97.0±0.45a	0±0.00b*	0.26±0.03
<i>Penicillium citrinum</i>	100±0.00a	99.3±0.96a	100±0.00a	99.9±0.04a	45.6±7.03b*	51.28±6.60

^{a,b,c} The same letters in each line indicate no significant differences among the data at 5% level by Tukey test. Values are the mean ± standard deviation: average of two experiments carried out in three replicates; -, No fungal growth; *Values of spore number were significantly different by the Tukey test when compared with the control (without bacteria).

3.3. Bacterial effect on spore germination

Due to the best results previously presented, *Bacillus* sp. P1 and *Bacillus* sp. P11 were tested for effect on the spore germination rate. *B. subtilis* ATCC 19659 was also assessed as control. After 24 h incubation, most of tested fungi spores without bacterial cells (positive control) germinated (Figure 2). However, the germination of spores in the presence of antagonistic bacteria was strongly influenced ($P < 0.05$) when compared with control. Once again, *Aspergillus* sp. UCO2A proved to be the most sensitive fungus because the conidial germination was not observed (100% reduction). The second best result was obtained for *A. flavus*, which presented an inhibition rate of 95.8 and 97.2% for *Bacillus* sp. P1 and *Bacillus* sp. P11, respectively. Similar results were found for the other fungal isolates examined (Figure 2). Treatments with *B. subtilis* ATCC 19659 did not significantly affect spore germination when compared with control.

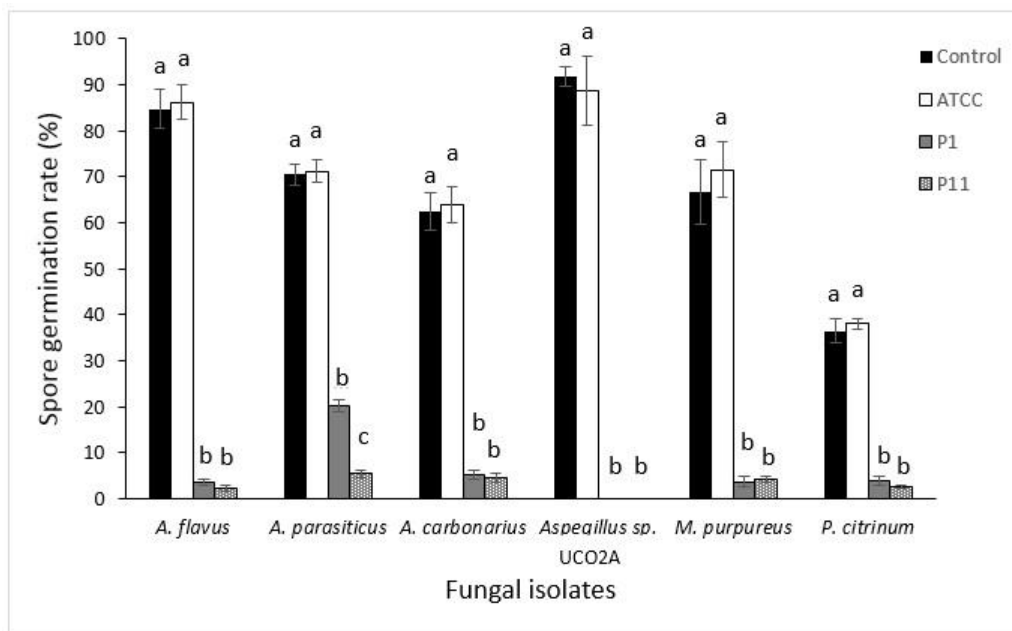


Fig. 2. Effect of *Bacillus* spp. on spore germination of toxigenic fungi after 24h incubation at 25°C on YES broth. Within each grouping, values with a letter in common are not significantly different according to Tukey test ($P < 0.05$). Error bars represent standard deviation of germination rate means.

3.4. Effect of *Bacillus* strains on mycotoxin production

Among the properties of biocontrol agents against toxigenic fungi, it is important to assess the effect on production of mycotoxins. As indicated in Table 6, the maximum level of AFB1 and OTA production for *A. flavus* and *A. carbonarius* under control conditions (without bacteria), were 535 and 67.5 µg/g, respectively. On the other hand, the *Bacillus* strains inhibited the mycotoxins production of target fungi when both organisms were grown simultaneously in PDA medium. Both *Bacillus* sp. P1 and *Bacillus* sp. P11 significantly reduced the AFB1 synthesis by 99.8% compared to the control. The percentages of OTA reduction by these strains were 97.5 and 97.3%, respectively. *B. subtilis* ATCC 19659 was able to reduce AFB1 and OTA production by 69.9 and 89.2%, respectively (Table 6). It could be observed that the bacterial strains had a reducing effect on ratio of mycotoxins per colony diameter. Figures 3 and 4 show the bacterial effects on mycotoxins production by fungal isolates, determined by HPLC analysis.

Table 6

Effect of *Bacillus* strains on aflatoxin B1 (AFB1) and ochratoxin (OTA) production by *Aspergillus* isolates growing on BDA plates at 25°C for 10 days. Values are expressed in µg/g of medium.

Treatments	AFB1 production (µg/g) by <i>A. flavus</i> *	OTA production (µg/g) by <i>A. carbonarius</i> *
Control	535.5±5.21a	67.5±8.4a
<i>B. subtilis</i> ATCC 19659	160.8±6.31b	7.2±0.61b
<i>Bacillus</i> sp. P1	0.98±0.16c	1.6±0.22c
<i>Bacillus</i> sp. P11	0.60±0.55c	1.8±0.23c

* Values are the mean; ±, Standard deviation: average of two experiments carried out in three replicates. The same letters in each column indicate no significant differences among the data at 5% level by Tukey test.

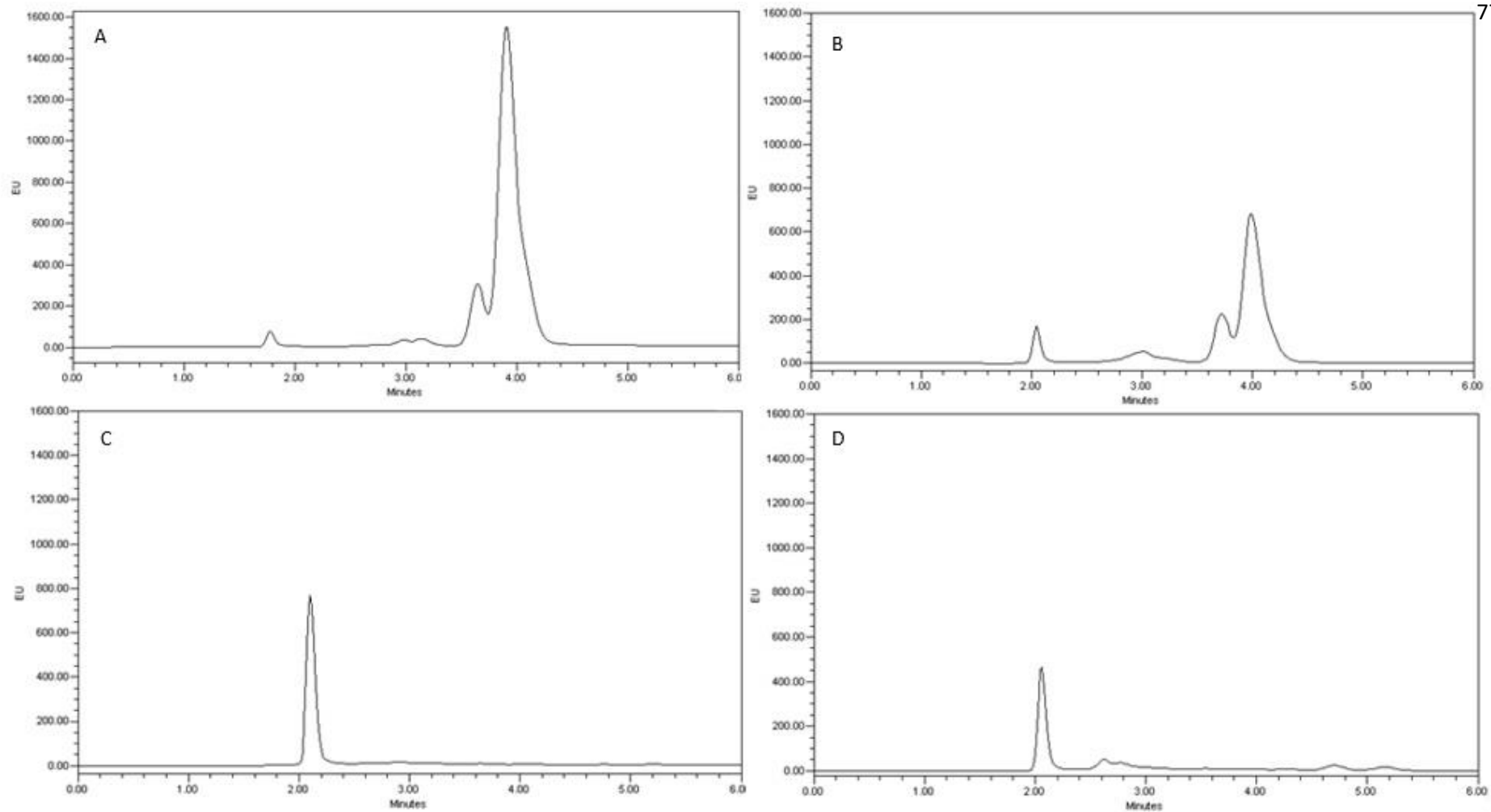


Fig. 3. High performance liquid chromatographic analysis of *Bacillus* strains effect on aflatoxin B1 production by *Aspergillus flavus* A12 cultivated on PDA medium at 25°C for 10 days. Mycotoxin production by fungal isolate cultivated alone (A), with *B. subtilis* ATCC 19659 (B), *Bacillus* sp. P1 (C) and *Bacillus* sp. P11 (D). AFB1 corresponds to the peak eluted at 3.9 min.

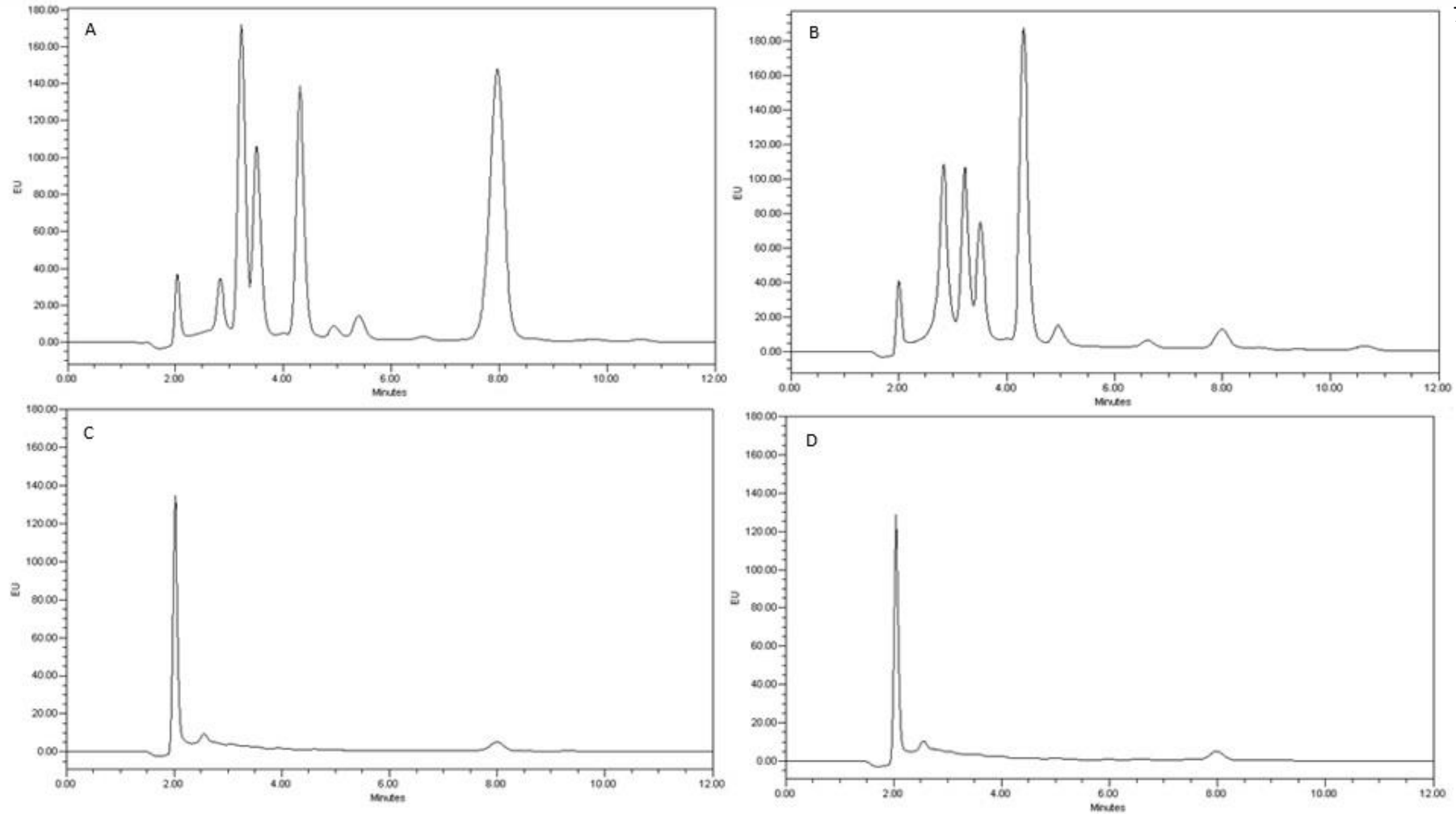


Fig. 4. High performance liquid chromatographic analysis of *Bacillus* strains effect on ochratoxin A production by *Aspergillus carbonarius* cultivated on PDA medium at 25°C for 10 days. Mycotoxin production by fungal isolate cultivated alone (A), with *B. subtilis* ATCC 19659 (B), *Bacillus* sp. P1 (C) and *Bacillus* sp. P11 (D). OTA corresponds to the peak eluted at 8.0 min.

3.5. Lipopeptides production by *Bacillus* spp.

The production of lipopeptides by *Bacillus* sp. P1, *Bacillus* sp. P11 and *B. subtilis* ATCC 19659 was checked by cultivation in BHI broth for 48 h before HPLC analysis. The results obtained demonstrated that *Bacillus* sp. P1 and *Bacillus* sp. P11 were capable to produce iturin A (Table 7). In addition, these bacteria produced three main isoforms of surfactin (C-13, C-14 and C-15 surfactin). Butanolic extracts from culture supernatants showed the highest quantity of these compounds. Based on comparing the retention time with the standards, the concentration of iturin A (601.9 mg/l) and C-15 surfactin (805.9 mg/l) produced by *Bacillus* sp. P1 was clearly higher than the concentration of these peptides from *Bacillus* sp. P11 (167.9 and 361.9 mg/l, respectively). The butanolic extracts caused a clear inhibition of *A. flavus* and *A. carbonarius* (Figure 5).

On the other hand, extracts from cell pellets showed lowest lipopeptide concentration, but in this case, the highest quantities of C-14 and C-15 surfactins was associated to *Bacillus* sp. P11, whose production reached values of 43 and 184.2 mg/l, respectively (Table 7). Further, C-13 surfactin was not detected in any cell pellet of the strains tested. None of these lipopeptides were found in extracts from *B. subtilis* ATCC 19659.

Table 7

Lipopeptides quantification produced by *Bacillus* strains. Butanolic extracts were obtained after 48h bacterial growth on BHI broth at 37°C, 125 rpm. The quantification was performed for iturin A and surfactin homologous, both for intra and extracellular compounds.

<i>Bacillus</i> strains	Cell-associated lipopeptides (mg/l)*				Extracellular lipopeptides (mg/l)*			
	Iturin A	Surfactin			Iturin A	Surfactin		
		C13	C14	C15		C13	C14	C15
ATCC	0±0.0c	0±0.0a	0±0.0c	0±0.0c	0±0.0c	0±0.0c	0±0.0c	0±0.0c
P1	6.21±1.5a	0±0.0a	16.5±0.4b	102.7±0.1a	601.9±15.1a	302±31.7a	477.1±13.1a	805.9±4.2a
P11	3.98±0.1b	0±0.0a	43±0.1a	184.2±1.2b	167.9±9.8b	107.5±6.5b	183.0±10.2b	361.9±2.7b

* Values are the mean; ±, Standard deviation: average of two experiments carried out in three replicates. The same letters in each column indicate no significant differences among the data at 5% level by Tukey test.

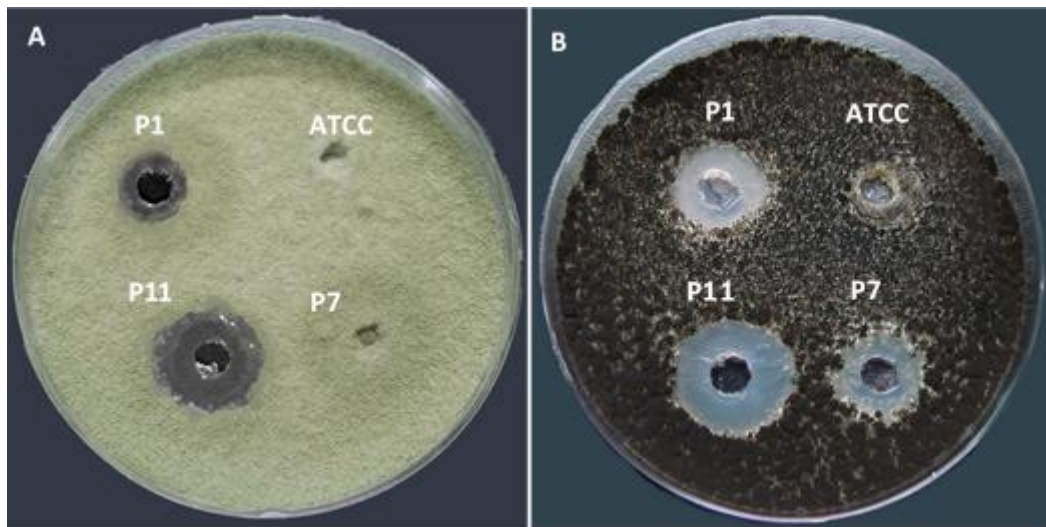


Fig. 5. Inhibitory activity of butanolic extract containing lipopeptides produced by *Bacillus* strains after growing on BHI broth at 37°C, 125 rpm for 48h. Aliquots (20 µl) of each extract were applied on wells of PDA plates containing 10⁶ spores/ml of the *Aspergillus flavus* (A) and *Aspergillus carbonarius* (B) isolates.

4. Discussion

Different *Bacillus* strains isolated from fish intestines were investigated to select bacteria presenting antagonism against toxigenic fungi. The tested bacteria presented inhibition zones predominantly characterized as moderate (clear zones from 3 to 9 mm) on traditional dual culture assay on PDA medium (Bacon and Hinton, 2002). Nevertheless, these values are within the range found in other studies of the genus *Bacillus* and other bacteria against several fungi (Bacon and Hinton, 2002; Bacon et al., 2006; Benitez et al., 2010). Although Velho et al. (2011) have demonstrated antifungal activity of some *Bacillus* strains against phytopathogenic fungi, the results obtained in this study are very relevant, since this is the first report on the inhibition of mycotoxin-producing fungi by these bacteria. Furthermore, strains P1 and B312 that also showed promising results have never been tested against any fungus. Afsharmenash et al. (2014) tested *B. subtilis* isolated from pistachio nuts against aflatoxigenic *A. flavus*, showing inhibition zone between 5.3 - 13.6 mm. Djossou et al. (2011) evaluated the antagonistic potential of various lactic acid bacteria against two isolates of *A. carbonarius* producers of OTA achieving a wide range of inhibition,

including the absence of activity. The *Bacillus* strains showed a broad spectrum of inhibition against the majority of target organisms. Hence, the screening test allowed to select four potential antagonistic bacteria, two of them already tested against non-toxicogenic fungi (*Bacillus* sp. P7 and *Bacillus* sp. P11) and the others (*Bacillus* sp. P1 and *Bacillus* sp. B312) never studied before.

These four bacteria were tested in order to determine the effects on mycelial growth, colony characteristics and sporulation of toxigenic fungi. These parameters are essential for any studies involving the biological control of fungi. All selected bacterial strains had an inhibitory effect on mycelial growth (over 70% reduction). Some authors have demonstrated a reduction on mycelial growth of toxigenic fungi by using antagonistic microorganisms, but it can be seen that different growth conditions may affect the results. Nesci et al. (2005) showed that *B. subtilis* had different responses in inhibiting *A. flavus* grown at two water activity levels (0.982 and 0.955). The simultaneous cultivation of *A. carbonarius* and *Saccharomyces cerevisiae* resulted in 50% reduction of mycelial growth at 25°C but complete inhibition was obtained by cultivation at 37°C (Armando et al., 2013). Therefore, it is possible to explore different growth conditions to obtain better results in the reduction of fungal growth, varying some factors such as cultivation temperature, water activity, composition and pH of culture medium.

Regarding the ability of *Bacillus* strains to inhibit the production of fungal spores, results are promising once synthesis of mycotoxins can be associated with sporulation (Brodhagen and Keller, 2006). On few occasions, high levels of mycotoxins can be found even when the spore production is inhibited (Adam and Yu, 1998; Mossini et al., 2009). However, another aspect of sporulation reduction is the limitation of fungal spread by lowering the spore load in the atmosphere and surfaces that are susceptible to contamination (Tzortzakis and Economakis, 2007). Inhibition of spore production that has occurred in the present experiment can be attributed to conidiogenesis process, since the fungi that produced few spores showed morphological changes in asexual structures, like elongated, whitish and/or poorly developed conidiophores compared to control (data not shown). Many reports have shown that *Bacillus* species produce antifungal compounds, which can affect the morphological development of fungal structures including some of these previously mentioned abnormalities (Fiori et al., 2014; Gong et al., 2014).

Studies on control of pathogenic fungi indicate that antifungal compounds produced by *Bacillus* species may also prevent conidia germination due to cytological alterations, particularly in cell wall and membrane (Moyne et al., 2001; Romero et al., 2007b; Zhang et al., 2008). Gong et al. (2014) showed that bacillomycin D, a lipopeptide of iturin group secreted by *B. subtilis*, affects both the mycelial growth as well as spore production and germination. Compounds of the iturin group can act on the cytoplasmic membrane of microorganisms, more specifically on the sterols (Quentin et al., 1982; Afsharmanesh et al., 2014). The results obtained in this study also show the ability of *Bacillus* spp. to reduce spore germination, but no morphological changes were observed under light microscope. It was not possible to observe any attachment to non-germinated spore, which is considered another mechanism of action of some bacteria on spore germination inhibition (Calvo et al., 2007). Thus, the possibility that the reduction of conidia germination occurs by producing antifungal compounds can not be ruled out.

The *Bacillus* species studied in this work significantly influenced the mycelial growth rate, percent of sporulation and spore germination rate of six mycotoxin-producing fungal isolates. Thus, another important fact to be considered is the bacterial influence on mycotoxin production. According to Gareis and Ceynowa (1994), mycotoxin synthesis can be induced by stress factors such as the presence of other microorganisms or chemical compounds, which may reduce the mycelial growth and sporulation, but in some cases, might stimulate the production of mycotoxins (Adam and Yu, 1998; Arrotéia et al., 2007; Ghorbanian et al., 2008, Mossini and Kimmelmeier, 2008). Cavaglieri et al. (2005) demonstrated that *Bacillus* spp. from maize rhizosphere inhibited *F. verticillioides* growth in co-culture, although the authors commented that one of *Bacillus* isolates stimulated fumonisin B1 synthesis. Mossini et al. (2009) reported that the use of neem oily extract has decreased mycelium growth and sporulation of *P. verrucosum* and *P. brevicompactum* (OTA producers), but the same was not obtained for the OTA formation.

As previously mentioned, it is essential to emphasize that during selection of a biological control agent, when the target organism is a toxigenic fungus, the effect on mycotoxin formation should be considered. Our findings revealed that *Bacillus* strains did not enhance either fungal growth or mycotoxin production (data relating to AFB1 and OTA). The production of these mycotoxins was reduced about 98% when the fungi were cultured simultaneously with bacteria. Similar results were obtained by

Munimbazi and Bullerman (1998), which reported a reduction in aflatoxin production by *A. parasiticus* ranging from 98.4 to 99.9% in the presence of *B. pumilus*. Bacterial influence on expression of genes involved in the synthesis of mycotoxins is one of the possible reasons for this reduction. For example, Kong et al. (2010), which observed that *B. megaterium* reduced the expression of *aflR* and *aflS* genes, the most important genes involved on aflatoxin biosynthesis, through altering on transcription.

The fungal isolates *A. flavus* and *A. carbonarius*, producers of higher concentrations of AFB1 and OTA (unpublished data), respectively, were selected to test the bacterial influence on mycotoxin production due to the increased risk of these mycotoxins (Marin et al., 2013; Ashiq et al., 2014; De Ruyck et al., 2015). The experiments were not designed to determine this effect on fungi producers of citrinin, despite the growth inhibition results obtained until now for *P. citrinum* and *M. purpureus* are extremely important. Although there are studies about inhibition *Penicillium* genus (Abraham et al., 2010; Wang et al., 2015). Currently, no research on biocontrol of citrinin producing fungi using the genus *Bacillus* has been reported. In the present study, it is possible that the bacteria reduced fungal growth before mycotoxin production (Mossini and Kemmelmeier, 2008). For that reason, further investigation is being developed in order to provide more information about antagonistic effect against citrinin producers isolates.

Concerning the mechanism involved in the biocontrol process of toxigenic fungi by *Bacillus* strains, Torres et al. (2016) comment that the mode of action of antagonistic microorganisms can affect pathogens by parasitism, food and space competition or production of toxic metabolites. These mechanisms may also be mentioned for *Bacillus* species. Based on the results obtained here, a first suggestion for the inhibitory mechanism could be by competition, since bacterial attachment to fungal structures was not observed for classification as parasitism. In addition, a preliminary test with cell-free supernatant in PDA medium containing spore suspension did not exhibit antifungal activity (data not shown). However, Velho et al. (2011) investigated the antifungal activity of some of these strains and confirmed the presence of genes involved in iturin and surfactin synthesis, indicating that the production of antifungal lipopeptides can not be ruled out.

The absence of inhibitory activity in the supernatants can be explained by the low concentration of antifungal substances. Nevertheless, after performing a bacterial growth followed by lipopeptides extraction from supernatant, the presence of iturin A

and surfactin isomers has been confirmed. The lipopeptides extraction procedure has increased their concentration, providing clear inhibition zone formation in additional tests with these extracts. This fact can be compared with the results obtained by Gong et al. (2006), which tested the biological control of phytopathogenic fungi using *B. subtilis*. Those authors found that the antifungal activity of extracted product from cultivation medium was 30 times more efficient than the supernatant one.

Bacillus species have been reported to produce a wide spectrum of lipopeptide compounds such as bacillomycins, iturins, mycosubtilin, fengycins, surfactins and similar substances (Thasana et al., 2010; Slivinski et al., 2012; Pretorius et al., 2015). Iturin A is one of the best-known lipopeptides produced by *Bacillus* genus, due to its potential as biocontrol agent of fungi (Gong et al., 2006; Afsharmanesh et al., 2014). Surfactins have been recognized as effective biosurfactants, showing synergistic antimicrobial action with other lipopeptide compounds (Maget-Dana, et al., 1992; Ongena and Jacques, 2008). Recent studies showed that different lipopeptides can also simultaneously produced by *Bacillus* spp. (Benitez et al., 2010; Ayed et al., 2015, Inès and Dhouhaa, 2015). It is possible that our bacterial strains produce a mixture of such compounds, because even though *Bacillus* sp. P1 obtained the highest concentrations of iturin A and surfactin isomers, did not present the best antifungal activity profile when compared with other bacteria. Thus, it is possible that multiple lipopeptides are involved in the biological control by *Bacillus* strains.

This study revealed that some *Bacillus* spp. obtained from fish intestines have antagonistic activity against fungi producers of AFB1, OTA and citrinin. In particular, *Bacillus* sp. P1 and *Bacillus* sp. P11 showed better results on reduction of mycelial growth, colony growth rate, number of fungal spores and spore germination rate. There is also evidence that the activity of these bacteria inhibited the production of AFB1 and OTA for the target fungi when compared to control treatment. It is quite possible that the antifungal activity is because of lipopeptide production, once the *n*-butanol extract from bacterial cultivation medium exhibited high concentration of iturin A and surfactins as well as confirmed the *in vitro* inhibition activity against fungal isolates.

The results of this work suggest that these bacterial strains are favorable candidates as a biocontrol agent of toxigenic fungi due to their capacity to reduce the fungal growth parameters and the levels of mycotoxins formed (AFB1 and OTA). Further studies are needed to improve the production of antifungal metabolites, to identify other compounds with the same activity and to elucidate the mode of action in

order to obtain more effective results. To our knowledge, this is the first report on inhibition of toxigenic fungi by *Bacillus* strains isolated from fish intestines of Amazon basin. This significantly contributes to knowledge of microbial and metabolic diversity of this highly heterogeneous region, and encourages the search and discovery of biological resources to fungal control.

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Supplementary Figure

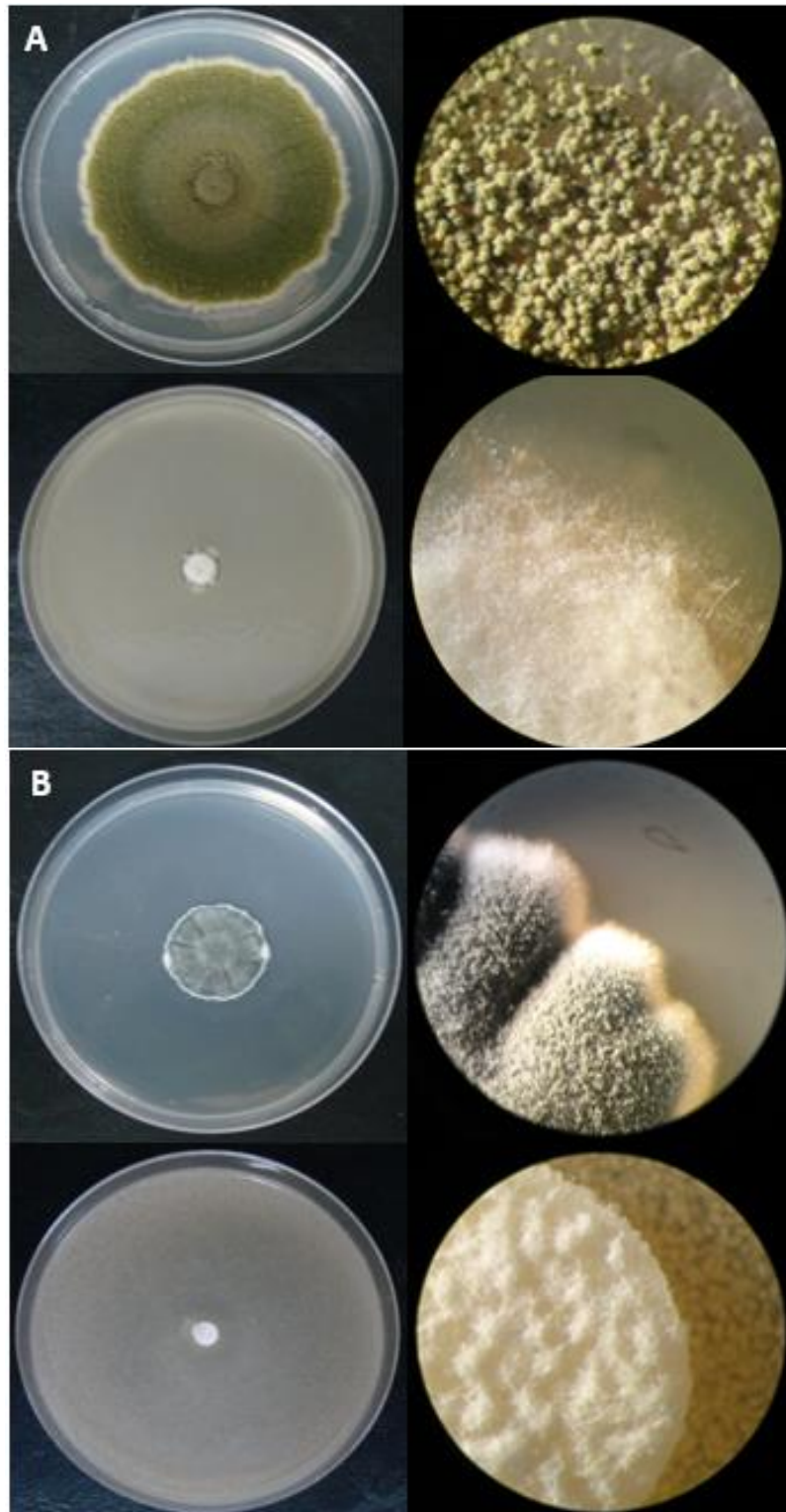


Figure S1. Mycelial growth of *Aspergillus parasiticus* (A) and *Penicillium citrinum* (B) after 10 days of incubation at 25°C on PDA plates without any bacteria (top) and co-cultured with *Bacillus* sp. P11(10^7 CFU/ml) (bottom). On the right, visualization by stereomicroscope (16x).

CAPÍTULO 3

Use of *Pseudomonas* sp. 4B isolated from aquatic environment as antagonist of toxigenic fungi

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Abstract

Aims: To investigate the antagonistic activity of *Pseudomonas* sp. 4B isolated from the effluent pond of a bovine abattoir against toxigenic fungi. This assessment approached the bacterial influence on fungal growth parameters and mycotoxin production.

Methods and Results: The antifungal activity of this *Pseudomonas* sp. 4B was studied through antibiosis assays against thirteen toxigenic fungi. This strain was able to form an inhibitory growth zone against all fungi tested. The influence on fungal growth parameters was assessed by simultaneous growing with the bacterium during 10 days. Bacterial cells reduced the diameter of fungal colonies, the colony growth rate and the sporulation of each indicator fungi. For spore germination test, known amounts of the bacterial suspension and fungal spores were added in Yeast Extract Sucrose broth for 48 h. The results proved satisfactory effects since reduced the conidial germination. *Pseudomonas* sp. 4B also influenced the production aflatoxin B1 and ochratoxin A for *Aspergillus flavus* and *A. carbonarius*, respectively.

Conclusion: The results show that *Pseudomonas* sp. 4B has a strong antagonistic action against toxigenic fungi and production of aflatoxin B1 and ochratoxin A.

Significance and Impact of the Study: The results demonstrated for the first time that a *Pseudomonas* strain isolated from aquatic environment had a great potential in controlling of toxigenic fungi.

Keywords

Fungal inhibition, biocontrol, mycotoxins, aflatoxin B1, ochratoxin A

1. Introduction

Fungi producers of mycotoxins are widely distributed in nature, mainly representatives of the genera *Aspergillus*, *Penicillium* and *Fusarium* (Pitt and Hocking 2009). Several studies have reported presence of these fungi in various agricultural commodities (Ruadrew et al. 2013; Oliveira et al. 2014; Ferre 2016; Phoku et al. 2016) and the occurrence of their mycotoxins in food and feedstuffs (Ferre 2016; Wambacq et al. 2016).

Mycotoxins are extremely toxic secondary metabolites produced by certain fungi and represent a huge impact for food safety due to acute toxic effects and/or chronic diseases that the ingestion of products contaminated by these compounds may induce. Among more than 400 mycotoxins, identified up to now, aflatoxin B1 (AFB1) and ochratoxin A (OTA) have been received particular attention due to hepatotoxic and nephrotoxic properties, respectively (De Ruyck et al. 2015). These fungal compounds exerts toxic effects at ng ml^{-1} concentrations and have cumulative effects on health (Lino et al. 2008; De Ruyck et al. 2015). Besides that, the presence of certain mycotoxins can also enhance the action of other ones, such as by synergism of OTA and citrinin (an emerging mycotoxin) (Gayathri et al. 2015). The International Agency for Research on Cancer classified AFB1 as carcinogen to humans (Group 1) (IARC 2012) and OTA as possible carcinogenic agent to humans (group 2B) (IARC 1993).

Several methods have been proposed to inactivate or detoxify mycotoxins in food. However, the high cost, efficacy and safety of many detoxification procedures are often questioned, as well as nutritional quality losses of the food (Fan *et al.* 2013; Gorran *et al.* 2013; Wambacq *et al.* 2016). Biological control strategies have received great attention because of the possibility of replacing the use of fungicides, which present risks to health and environment, and may results in the occurrence of fungicide-resistant fungi and/or increase the mycotoxins synthesis (Abraham *et al.* 2010; Kong *et al.* 2010; Sangmanee and Hongpattarakere 2014).

Many species and strains of the *Pseudomonas* genus produce a range of substances with antimicrobial action. Suppressive effect against certain filamentous fungi by pseudomonads isolated from various environments has been investigated (Thrane *et al.* 2000; Berry *et al.* 2010; Gorantla *et al.* 2014, Panebianco *et al.* 2015), including the ability of these bacteria to inhibit growth of aflatoxigenic fungi (Nesci *et al.* 2005; Palumbo *et al.* 2006; Akocak *et al.* 2015). In most cases, antifungal substances produced from *Pseudomonas* species were reported as oxylipins (Martin-

Arjol *et al.* 2010), viscosinamide (Thrane *et al.* 2000), phenazine (Chen *et al.* 2015), lipopeptides (Berry *et al.* 2010; Reder-Christ *et al.* 2012), among others. However, research about biocontrol of new *Pseudomonas* strains should be encouraged in order to broaden the activity spectrum against phytopathogenic and toxigenic fungi.

A study carried out by Fontoura *et al.* (2009) found that a bacterial strain, isolated from the effluent pond of a bovine abattoir and characterized as *Pseudomonas* sp. 4B, has shown inhibitory effect against a broad range of Gram-positive and Gram-negative bacteria. These authors also evaluated the bacterial effect on some yeasts: *Candida utilis*, *Kluyveromyces marxianus*, *Candida kefir* and *Malassezia paquidermathis*, but the inhibition was observed only in the first two yeasts. Besides that, this bacterium has not yet been investigated with regard the control of fungi, more specifically producers of mycotoxins. Based on that, this study aimed at evaluating the antagonistic activity of *Pseudomonas* sp. 4B against toxigenic fungi by addressing the effects of this strain on fungal growth parameters and mycotoxins production.

2. Material and methods

2.1 Microorganisms and preparation of the inocula

The strain *Pseudomonas* sp. 4B, belonging to the culture collection of the Laboratório de Bioquímica e Microbiologia Aplicada (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil), was used in the study. This bacterium was isolated from the effluent pond of a bovine abattoir localized at southern Brazil (Fontoura *et al.* 2009). Stock culture was maintained at -20°C in Brain Heart Infusion broth (BHI; Oxoid, Basingstoke, UK) with 20% (v/v) glycerol or stored at 4°C BHI agar plates. Before use, the *Pseudomonas* cells were propagated twice in the same medium at 37°C for 24 h. The bacterial inoculum was obtained from the working culture by incubation into 10 ml BHI broth on a rotary shaker (125 rev min^{-1}) during 24 h at 37°C . The cell concentration was determined by measuring the optical density at 600 nm and standard plate count and then adjusted to about 10^7 CFU ml^{-1} .

Toxigenic isolates of fungi used as indicator micro-organisms for antagonism assays are listed in Table 1. These fungal strains were maintained on potato dextrose agar (PDA; Acumedia, Lansing, MI, USA) slants covered with 20% glycerol at 4°C and routinely grown on PDA agar at 25°C . Spore inocula were prepared by growing the fungi on PDA plates for 7 days at 25°C . Aliquots of 5 ml of sterile distilled water with 0.05% (v/v) Tween 80 were poured on each of the colonies and then the spores were

removed with the aid of a loop of Drigalski. The conidial suspension was collected and transferred to a sterile tube and the spore count was then determined with the aid of a Neubauer chamber. The concentration of spore suspension was adjusted to 1×10^6 spores ml^{-1} , before performing experiments.

Table 1 Isolates of toxigenic fungi used as indicator micro-organisms for antagonism assays. The mycotoxins produced by these fungi are also displayed

Indicator fungi	Mycotoxin
<i>Aspergillus flavus</i> A12	AFB1 ^b
<i>Aspergillus</i> sp. A65	AFB1
<i>A. parasiticus</i> 30BL	AFB1
<i>A. carbonarius</i> ITAL 293 ^a	OTA ^c
<i>Aspergillus</i> sp. UMO1B	OTA
<i>Aspergillus</i> sp. UCO1A	OTA
<i>Aspergillus</i> sp. UCO2A	OTA
<i>Aspergillus</i> sp. UCS2B	OTA
<i>Penicillium citrinum</i> ITAL197 ^a	citrinin
<i>Monascus purpureus</i> NRRL1992	citrinin

^aIsolates kindly provided by Instituto de Tecnologia de Alimentos (ITAL), São Paulo, Brazil; ^bAFB1: aflatoxin B1; ^cOTA: ochratoxin A.

2.2 Antifungal assays

In order to evaluate antagonistic activity of *Pseudomonas* sp. 4B against the toxigenic fungi, *in vitro* assays were performed according to Benitez *et al.* (2010) with some modifications. The sterile molten PDA at 45°C was mixed with fungal spore suspension (final concentration 1×10^6 spores ml^{-1}). The medium was plated in Petri plates and after solidifying, 20 μl of bacterial liquid culture (10^7 CFU ml^{-1}), early growing on BHI broth at 37°C and 125 rev min^{-1} for 48 h, was inoculated on the surface of each plate. The cell-free culture supernatant was also tested. In this case, cells were separated by centrifugation at 10 000 **g** for 15 min at 4°C and the culture supernatant was sterilized by filtration with 0.22 μm cellulose membranes (Millipore, Bedford, USA).

After incubation for 5 days at 25°C, the antagonism was indicated by the formation of inhibition zones whose width was measured using a digital caliper (values in mm). The following score was used to classify antagonistic abilities of *Pseudomonas*

sp. 4B: -, no antagonism; +, weak antagonism (inhibition zones < 3 mm); ++, moderate antagonism (inhibition zones \geq 3 - 9 mm); +++, strong antagonism (inhibition zones > 9 mm).

2.3 Bacterial effect on fungal growth

The effects of *Pseudomonas* cells on fungal growth, colony characteristics and sporulation of two representative producers of AFB1, OTA or citrinin were assessed. This method was adapted from Nesci *et al.* (2005) and Gandomi *et al.* (2009). Bacteria cells were obtained by centrifugation at 10 000 **g** for 15 min after growing as previously described. Fifteen milliliters of sterile molten PDA containing bacterial cells suspension (10^7 CFU ml⁻¹) were transferred to Petri dishes (80 mm diameter) and allowed to solidify. After that, a sterile paper disc (5 mm diameter) was placed at the center of agar surface and 10 μ l of fungal spore suspension (10^6 spores ml⁻¹) were spotted onto the discs. The control plates were inoculated with test fungi but without bacteria. All Petri dishes were then incubated at 25°C for 10 days. Diameters values of fungal colonies were taken in two directions at right angles to each other during this time and used to plot growth curves.

Percentage inhibition of mycelial growth was determined by comparing the growth in the plates containing both micro-organisms with the control cultures and calculated according the following equation:

$$\text{Inhibition of growth (\%)} = ((D_c - D_t) / D_c) \times 100.$$

In this case, D_c represents the diameter of fungal colony in control plate and D_t the diameter of fungal colony in treated plate containing the antagonist bacterium.

When the hyphae in the control plate reached the maximum diameter of the plate (80 mm) or after 10 days, the colony growth rate (mm day⁻¹) of each plate was determined by linear regression of the linear phase for growth.

After growth was evaluated, all samples were analyzed for bacterial influence on fungal spore production. The spores produced were washed by 15 ml Tween 80 solution (0.05%) and the estimate of conidial concentration (spores cm² of colony) was performed using a Neubauer chamber. The following formula was employed in order to calculate the percent of sporulation inhibition:

$$\text{Inhibition of sporulation (\%)} = ((N_c - N_t) / N_c) \times 100.$$

The number of fungal spores produced in control and treated plates is represented by N_c and N_t , respectively. Treatment means presence of the antagonist bacterium.

2.4 Spore germination inhibition test

In order to evaluate inhibitory activity of *Pseudomonas* sp. 4B on the fungal spore germination, *in vitro* assays were performed according a procedure adapted from Wang et al. (2008) and Gameda et al. (2014). Fungal spore suspensions (10^6 spores ml^{-1}) were prepared as described earlier and $100 \mu l$ of each suspension was transferred to sterile glass tubes containing $800 \mu l$ of YES broth (2% yeast extract and 20% sucrose; pH 6.5). Then, $100 \mu l$ of *Pseudomonas* cells (10^7 CFU ml^{-1}) was also added into the same tubes. Control treatment consisted of $100 \mu l$ sterile saline solution (0.85%) instead of bacterial cells and used to comparison.

After 24 h incubation at $25^\circ C$, conidia germination was evaluated by using a light microscope (Olympus CX40, Japan). Spores were regarded to have germinated when the length of germination tube was equal or longer than the diameter of its own spore. Four counts of 100 conidia per repetition were performed and the percent spore germination determined by dividing the number of germinated spores over the total number of spores counted. Percent spore germination inhibition was determined as follows:

$$\text{Inhibition of spore germination (\%)} = ((G_c - G_t) / G_c) \times 100.$$

In this equation, G_c represents the percentage of spore germinated in the control tube and G_t is the percentage of spore germinated in treated tube containing the test bacterium.

2.5 Mycotoxins production assays

The bacterial influence in production of the most important mycotoxins (AFB1 and OTA) was evaluated. In this case, only the largest producers of these toxins were selected. PDA plates containing bacterial cells and test fungi were prepared again as previously described on section about the bacterial effect on fungal growth. The mycotoxins were extracted from the medium after 10 days incubation at $25^\circ C$ following the method described by Nesci *et al.* (2005) with minor modification. The PDA medium (1 x 1 cm), including microbial biomass, was taken from the fungal colony of each treatment and transferred to a 2 ml Eppendorf tube. A $500 \mu l$ aliquot of chloroform

was added in the same tube and then this mixture was shaken at 100 rev min^{-1} during 30 min. After that, the chloroform extract was filtered through PTFE $0.22 \mu\text{m}$ syringe filter (Millipore, USA) and poured into a glass vial in order to dry under nitrogen gas. The residue was redissolved in $250 \mu\text{l}$ of the mobile phase as necessary for high performance liquid chromatography (HPLC) analysis. The extraction procedure was carried out twice from the same piece of agar medium.

Determination of mycotoxins was performed using an HPLC instrument (Waters, model E2695) equipped with a fluorescence detector (Waters, FL-2475). The separation, identification and quantification steps of AFB1 and OTA followed the methods described by Xu *et al.* (2013) and the Association of Official Agricultural Chemists (AOAC 2001), respectively. Separations were performed on a C18 column (XBridge™ Shield RP 18; $5 \mu\text{m}$; $4.6 \times 150 \text{ mm}$, Waters, Ireland) connected to a precolumn ($4.6 \times 20 \text{ mm}$) of the same model. The mobile phase employed was acetonitrile/water/acetic acid (99: 99: 2, v/v/v) under isocratic conditions. A $20 \mu\text{l}$ aliquot of the samples was eluted at a flow rate of 1.0 ml min^{-1} and experiment conducted at 30°C . The fluorescence detector was operated at 360 nm for excitation and 440 nm for emission on AFB1 analysis. Regarding OTA detection, the excitation and emission wavelengths were set to 333 and 460 nm, respectively.

Standard curves were done under these conditions with seven levels of standard mycotoxins obtained from Sigma (Sigma-Aldrich, Steinheim, Germany), ranging from 2.5 to $50 \mu\text{g l}^{-1}$ for AFB1 and 0.5 to $6 \mu\text{g l}^{-1}$ for OTA. The retention time of AFB1 and OTA was 3.9 and 6.5 min, respectively. Quantification of these toxins was performed by correlating chromatographic peak area of each toxin found in sample extracts with the respective straight-line equation obtained from calibration curves. Average recovery values of $98.9\% \pm 1.0$ and $99.0\% \pm 1.2$ for AFB1 (at level of $50 \mu\text{g l}^{-1}$ added into PDA medium) and OTA (at level of $6 \mu\text{g l}^{-1}$), respectively were found. The values for limit of detection (LOD) were 0.014 and $0.06 \mu\text{g l}^{-1}$ for AFB1 and OTA, respectively. The limit of quantification (LOQ) was $0.047 \mu\text{g l}^{-1}$ for AFB1 and $0.6 \mu\text{g l}^{-1}$ for OTA. Method validation, including recuperation rate, LOD and LOQ was performed according to International Conference on Harmonization (ICH, 1996).

2.6 Data analysis

All results were expressed as the means \pm SD (standard deviation) of three replicates. Analysis of variance (ANOVA) was performed for the data obtained using SAS for Windows ver. 9.0 (SAS Institute Inc., Cary, NC). Differences were reported at a significance level of 95% by Tukey Test.

3. Results

3.1 Antagonism *in vitro*

In this study, *Pseudomonas* sp. 4B was initially evaluated in relation to the antagonistic activity against toxigenic fungi after 5 days on PDA plates. The results obtained showed that this bacterium was considered antagonist due to formation of inhibition zones for all fungal isolates tested (Fig. 1). The best result was observed against *Penicillium citrinum* ITAL197 since *Pseudomonas* sp. 4B produced inhibition zone of 15.7 mm during the fungal growth (Table 2). The smaller inhibition zones were observed for the representatives of *Aspergillus* genus (1.3 to 2.3 mm). The cell-free culture supernatant has not obtained any activity against fungal isolates. From these results, two fungal isolates producers of AFB₁, OTA and citrinin were used in the following assay in order to observe the bacterial effect on growth parameters of these fungi.

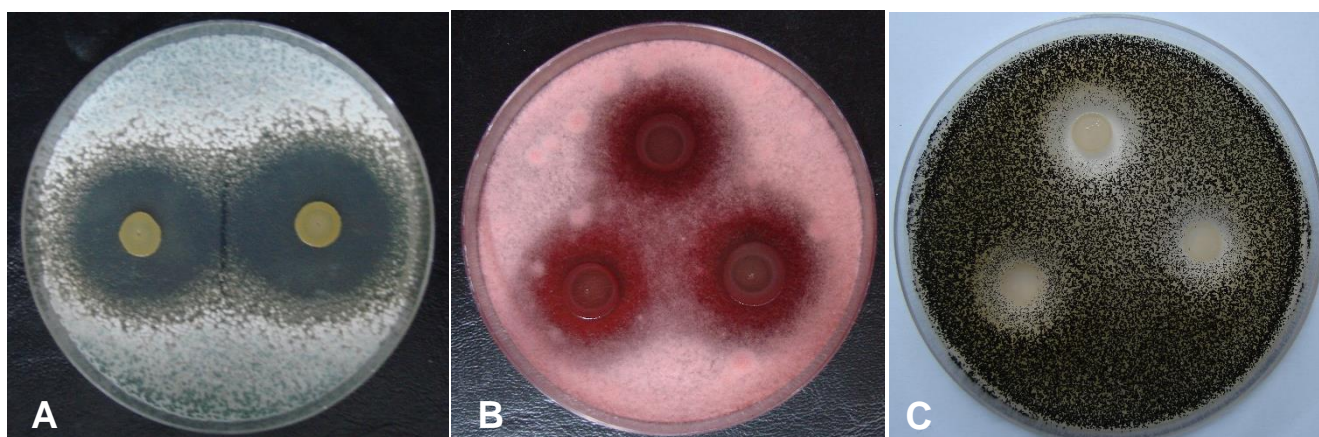


Figure 1 Inhibition halos on *Penicillium citrinum* ITAL197 (A), *Monascus purpureus* NRRL1992 (B) and *Aspergillus* sp. UCO2A (C) caused by *Pseudomonas* sp. 4B cells after 5 days of incubation at 25°C on PDA plates. The bacterial strain was growing on BHI broth at 37°C, 125 rev min⁻¹ for 48h before the test.

Table 2 *In vitro* antagonism of *Pseudomonas* sp. 4B against mycotoxin-producing fungi cultivated on PDA plates at 25°C for 5 days.

Fungal isolates	Antagonism	
	Cells	Supernatant*
<i>A. flavus</i> A12	+	-
<i>Aspergillus</i> sp. A65	+	-
<i>A. parasiticus</i> 30BL	+	-
<i>A. carbonarius</i> ITAL 293	+	-
<i>Aspergillus</i> sp. UMO1B	+	-
<i>Aspergillus</i> sp. UCS2B	+	-
<i>Aspergillus</i> sp. UCO2A	+	-
<i>Aspergillus</i> sp. UCO1A	+	-
<i>P. citrinum</i> ITAL197	+++	-
<i>M. purpureus</i> NRRL1992	+++	-

-, no antagonism; +, weak antagonism (clear zones of inhibition < 3 mm); ++, moderate antagonism (clear zones of inhibition \geq 3 - 9 mm); +++, strong antagonism (clear zones of inhibition > 9 mm). Data from three trials with three replicates per trial. * Cell-free culture supernatant.

3.2 Effect of *Pseudomonas* sp. 4B on growth and sporulation inhibition

Regarding the effect of *Pseudomonas* sp. 4B on fungal growth parameters, data are shown in Table 3. The effect of *Pseudomonas* sp. 4B on profile of mycelial growth according incubation time was shown in Figure S1 of Supporting Information. The bacterium was effective for significantly reduce ($P < 0.05$) mycelial growth when compared to the diameter of the fungal colonies in treatment control. In particular, *Pseudomonas* sp. 4B had greater influence on *Aspergillus* sp. UCO2A in which was able to reduce by 86.1% the fungal colony diameter. Besides that, the colony growth rate decreased from 3.42 to 0.25 mm day⁻¹ when this fungus was co-inoculated with *Pseudomonas* sp. 4B. Despite these results, the presence of bacteria did not reduce satisfactorily the sporulation. In this case, the number of fungal spores per square centimeter produced was reduced to only about 32%.

Representative results were also observed on the growth of *M. purpureus* NRRL1992 since the bacterial isolate reduced by 85.4% the diameter of the fungal colony thereby influencing on decrease of the colony growth rate (0.06 mm day⁻¹).

Furthermore, although there was a slight growth of the colony, sporulation was completely inhibited since reproductive structures were not present in microscopic view. In the same way, both *A. parasiticus* 30BL and *P. citrinum* ITAL197 showed: a 67.1% reduction in the colonies diameter, a very slow mycelial growth when compared to control and 100% inhibition of conidia production. Finally, *A. flavus* A12 was the fungal isolate more resistant to effect of *Pseudomonas* sp. 4B, only 43.3% reduction in growth. Even so, this bacterium was capable to significantly decrease ($P < 0.05$) the colony growth rate (from 4.12 to 2.15 mm day⁻¹) of *A. flavus* A12 and mainly to reduce the sporulation by almost 80%.

3.3 Inhibition of spore germination

Spores of toxigenic fungi were exposed to *Pseudomonas* cells for assessing some bacterial influence on their germination. *Pseudomonas* sp. 4B showed a strong inhibitory activity on the number of germinated conidia for all tested fungal isolates after 24 h incubation on YES broth (Fig. 2). The spore germination rate in the presence of antagonistic bacterium was significantly lower ($P < 0.05$) when compared with control (without bacterial cells), achieving values below 10%. Spores of *P. citrinum* ITAL197 were the most sensitive since the antagonistic bacterium inhibited completely (100%) the conidial germination. Besides, spore inhibition rate for the other fungal isolates ranged from 84.1 to 98.5% and no abnormality was observed microscopically in spores and germ tubes.

Table 3 Influence of *Pseudomonas* sp. 4B on growth parameters of toxigenic fungi after 10 days of incubation at 25°C on PDA plates. Fungi growing alone were considered as control

Fungal isolates	Mycelial growth		Colony growth rate (mm day ⁻¹)		Spore production	
	Inhibition of growth (%)	Control colony diameter (mm)	Treatment with bacterial cells	Control	Sporulation inhibition (%)	Control (spores x 10 ⁶ cm ²)
<i>A. flavus</i> A12	43.3±1.90	80.0±0.00	2.15±0.06	4.12±0.01	79.7±10.79	1.82±4.14
<i>A. parasiticus</i> 30BL	67.1±1.37	66.8±0.63	0.85±0.03	2.86±0.04	100±0.0	4.82±0.63
<i>A. carbonarius</i> ITAL 293	79.7±1.17	80.0±0.00	0.20±0.04	3.97±0.02	32.14±4.54	0.86±0.02
<i>Aspergillus</i> sp. UCO2A	86.1±2.20	80.0±0.00	0.25±0.07	3.42±0.03	32.9±2.42	0.08±0.02
<i>M. purpureus</i> NRRL1992	85.4±1.37	53.9±1.31	0.06±0.02	2.63±0.07	100±0.0	0.26±0.03
<i>P. citrinum</i> ITAL197	67.1±3.12	20.6±0.26	0.05±0.01	0.75±0.03	100±0.0	51.2±6.60

Values are the mean ± standard deviation: average of two experiments carried out in three replicates. Values of colony diameter, colony growth rate and spore number in the treatment with the bacterium were significantly different by Tukey test ($P < 0.05$) when compared with the control.

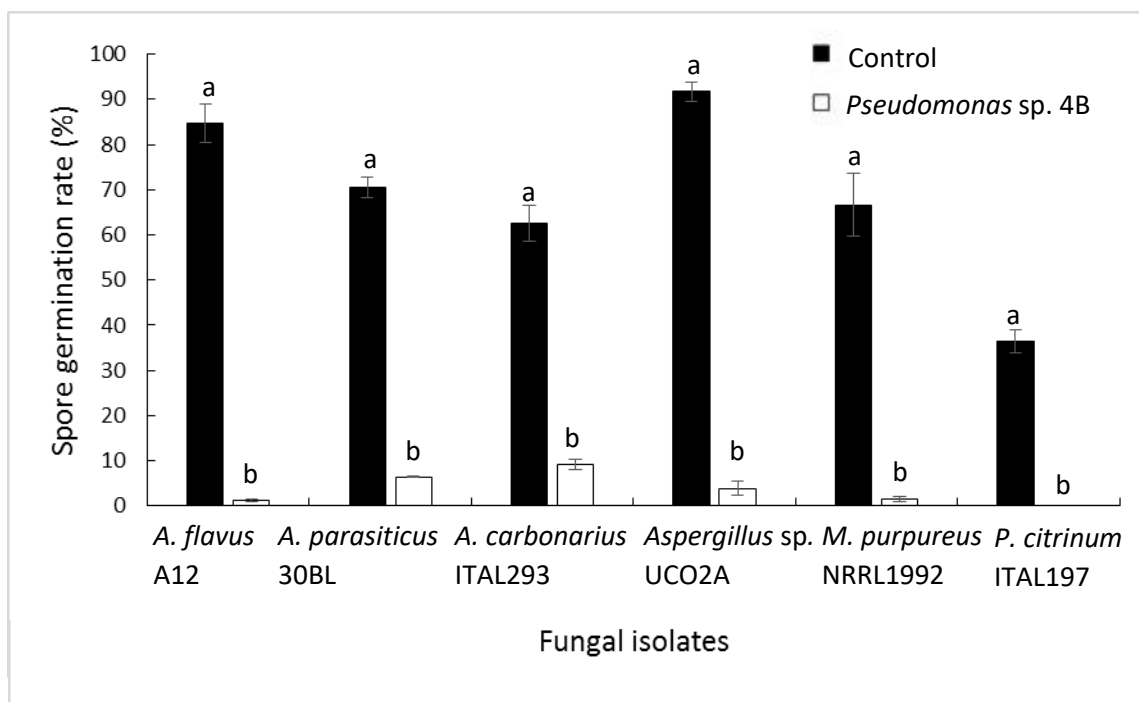


Figure 2 Effect of *Pseudomonas* sp. 4B on spore germination of toxigenic fungi after 24h incubation at 25°C on YES broth. Fungi growing alone were considered as control. Within each grouping, values with a letter in common are not significantly different according to Tukey test ($P < 0.05$). Error bars represent standard deviation of germination rate means.

3.4 Effect of *Pseudomonas* on mycotoxin production

The influence of *Pseudomonas* sp. 4B on the mycotoxins production was also investigated. *A. flavus* A12 and *A. carbonarius* ITAL293 growing without *Pseudomonas* (control conditions) produced 27.7 and 13.7 $\mu\text{g g}^{-1}$ for AFB1 and OTA, respectively. When the fungal isolates were co-cultivated with *Pseudomonas* cells a reduction in levels of these mycotoxins was observed. The percentages of AFB1 and OTA reduction by this bacterial strain were 94.2 and 63.8%, respectively. It could be observed that *Pseudomonas* sp. 4B had a reducing effect on ratio of mycotoxins per colony diameter. Results of the bacterial effect on mycotoxins production are presented in Table 4 and Figure S2 of Supporting Information.

Table 4 Effect of *Pseudomonas* sp. 4B on aflatoxin B1 (AFB1) and ochratoxin (OTA) production by *Aspergillus* isolates growing on BDA plates at 25°C for 10 days. Values are expressed in $\mu\text{g g}^{-1}$ of medium.

Treatments	AFB1 production ($\mu\text{g g}^{-1}$) by <i>A. flavus</i> A12	OTA production ($\mu\text{g g}^{-1}$) by <i>A. carbonarius</i> ITAL293
Control	27.7 \pm 5.82a	13.7 \pm 2.52a
<i>Pseudomonas</i> sp. 4B	1.58 \pm 0.60b	4.97 \pm 0.78b

Values are the mean \pm standard deviation: average of two experiments carried out in three replicates. The same letters in each column indicate no significant differences among the data at 5% level by Tukey test.

4. Discussion

The results of our study are very relevant because demonstrated for the first time that *Pseudomonas* sp. 4B had great potential to inhibit filamentous fungi growth, including mycotoxin producers. The formation of inhibition zones in all evaluated fungi was considered as indicative of the fungal sensitivity to the action of bacterial cells present in the same medium. Several *in vitro* studies have shown the formation of inhibition halos in filamentous fungi due to the presence of *Pseudomonas* species (Nagarajkumar *et al.* 2004; Naik and Sakthivel 2006; Berry *et al.* 2010; Cordero *et al.* 2012; Morohoshi *et al.* 2013; Panebianco *et al.* 2015). Similar results were also reported in other studies using different micro-organisms as biological control agents of phytopathogenic and toxigenic fungi (Bacon *et al.* 2006; Tupinambá *et al.* 2008; Djossou *et al.* 2011; Velho *et al.* 2011; Sangmanee and Hongpattarakere 2014).

This study also represents the first detailed report about the influence of this bacterial strain in growth parameters (mycelial growth, colony characteristics and sporulation) of toxigenic fungi. These parameters are essential for any studies involving the biological control of fungi.

Satisfactory effect has been obtained in vitro assay of co-inoculation (fungus–bacterium) on PDA plates. The bacterial strain could not completely inhibit fungal growth, but the values of colony diameter reduction obtained in our research (43-86%

in relation to control assay) were more promising than other studies carried out using species of the *Pseudomonas* genus against phytopathogenic fungi. For example, Manikandan *et al.* (2010) found that a *P. fluorescens* strain reduced the mycelial growth of *A. solani* e *F. oxysporum* by 44.4 and 40%, respectively. On the other hand, 14 *Pseudomonas* spp. strains showed mycelial reduction of *P. digitatum* ranging from 17.1 to 38.2% (Panebianco *et al.* 2015). Other antagonists such as *Bacillus amyloliquefaciens*, *Burkholderia cepacia* (Dihazi *et al.* 2012), *B. subtilis*, *Brevibacterium linens* (On *et al.* 2015) and yeasts (Bleve *et al.* 2006; Armando *et al.* 2013) displayed similar results.

Pseudomonas sp. 4B was also capable of influencing the spore formation in most of tested fungi. The complete sporulation inhibition for *A. parasiticus* 30BL, *M. purpureus* NRRL1992 and *P. citrinum* ITAL197 could be seen as an advantage for the control of toxigenic fungi since usually mycotoxins production can be associated with sporulation (Brodhagen and Keller 2006). Few studies have reported higher mycotoxin production when there is no sporulation (Adam and Yu 1998; Mossini *et al.* 2009). Curiously, the bacterial strain caused lower reduction in the colony diameter of *A. flavus* A12, but in contrast decreased significantly the number of spores. In this case, the result is promising due to lower possibility of spore dissemination in the atmosphere and surfaces that are susceptible to contamination (Tzortzakis and Economakis, 2007).

Concerning the bacterial effect on spore germination, *Pseudomonas* sp. 4B also proved considerable inhibitory activity after 24 h of contact with fungal spores. Studies have demonstrated different data about inhibition of conidial germination by *Pseudomonas* species (Wang *et al.* 2005; Akocak *et al.* 2015; Panebianco *et al.* (2015). Akocak *et al.* (2015) have found that *P. fluorescens* cells did not affect the spore germination of an aflatoxigenic *A. flavus* strain, but a slight reduction of only 20% was observed when spores were inoculated with an extracellular chitinolytic enzyme extract of this bacterium. The same authors suggest that different responses in spore germination assays could be due to species-specific targeting of each bacteria tested. In contrast, Panebianco *et al.* (2015) evaluated the inhibition of spore germination of *Penicillium digitatum* using cells of *Pseudomonas syringae* strains isolated from herbaceous and woody plant. After 24 h incubation, a germination reduction ranging

from 76.9 – 100% was observed, but the cell concentration (10^9 CFU ml⁻¹) and its volume (250 μ l) applied were greater than in our experiments.

In the present study, damage in cell wall and membrane of ungerminated spores were not visualized by light microscopy. Nevertheless, this fact does not exclude the possibility of antifungal compounds production by *Pseudomonas* sp. 4B. since metabolic adaptations that prevent spores may occur in the presence of harmful compounds (Uldahl and Knutsen 2009) blocking the germination process. Besides, morphological changes of cellular organelles alterations must be analyzed in detail through scanning and transmission electron microscopy to ensure the absence of damage.

Finally, the effect of coculturing this bacterial strain with *A. flavus* A12 or *A. carbonarius* ITAL293 exhibited some influence on production of AFB1 and OTA, respectively. These two strains were selected due to higher production of their mycotoxins in preliminary assays (unpublished data), furthermore of the greatest number of reports about food contamination by these species and the toxic effects of AFB1 and OTA (De Ruyck *et al.* 2015; Ferre 2016). The HPLC results from fungi cultured simultaneously with *Pseudomonas* sp. 4B have displayed a reduction in production of AFB1 or OTA. Our findings regarding the decrease of AFB1 production are consistent with those reported by Nesci *et al.* (2005). These authors found that *Pseudomonas solanacearum* inhibited AFB1 formation by *A. flavus* and *A. parasiticus* strains, with reduction ranging from 56 – 100%. About the OTA production, despite a lower bacterial influence, our data are critically important since the presence of other micro-organisms or chemical compounds that reduce the fungal growth is considered as a stress condition. Thus, in some cases, stress factors might stimulate the synthesis of mycotoxins (Adam and Yu 1998; Arrotéia *et al.* 2007; Ghorbanian *et al.* 2008, Mossini and Kemmelmeier 2008; Ahmed *et al.* 2015).

Several factors may be involved on inhibition of mycotoxins production when fungi were co-cultivated with bacterium. According Munimbazi and Bullerman (1998), competition for space and essential nutrients for the synthesis of aflatoxin as well as metabolites production by co-existing *Bacillus* could to play a certain role on aflatoxins formation, which may influence on expression of genes involved in the synthesis of mycotoxin.

Since this work has focused on influence of *Pseudomonas* sp. 4B in AFB1 and OTA production, the experiments were not designed to determine this effect on fungi producers of citrinin. The growth inhibition results obtained for *P. citrinum* ITAL 197 and *M. purpureus* NRRL1992 are extremely important because there are few reports on antagonism of *Pseudomonas* species against potential citrinin producing fungi such as *P. expansum*, *P. viridicatum*, *P. citrinum* and *A. oryzae*. Besides that, the fungi of these studies did not confirm their ability to produce citrinin (Wang *et al.* 1999; Cirvilleri *et al.* 2005; Gorantla *et al.* 2014).

Additionally, the antagonist effect of *Pseudomonas* sp. 4B was assessed for other fungi such as *P. chrysogenum* and *Fusarium graminearum* (unpublished data), producers of mycotoxins that less reported than AFB1 and OTA, for example, roquefortine C and fusarenon X. However, these toxins, as well as citrinin, can also be found in foods and may present neurotoxic, nephrotoxic and immunosuppressive properties (Saengtienchai *et al.* 2014; Fontaine *et al.* 2015). Because of this, studies are now in progress to provide more information about antagonistic effect of *Pseudomonas* sp. 4B against fungi producers of emerging mycotoxins.

Pseudomonas sp. 4B achieved satisfactory results on all parameters assessed which are essential for any studies involving the biological control of toxigenic fungi. In considering some modes of action of antagonistic micro-organisms (parasitism, competition or production of toxic metabolites), the absence of bacterial attachment to fungal structures excludes any possibility of parasitism. Competition for space and nutrients could be associated to the data presented, mainly due to the absence of damage to cell structures, however specific competition assays, such as index of dominance and niche overlap indices tests, are necessary to support this claim (Nesci *et al.* 2005; Bleve *et al.* 2006).

Test with cell-free supernatant in PDA medium did not exhibit antifungal activity. Nevertheless, the production of antifungal compounds can not be ruled out. Fontoura *et al.* (2009) attributed the antimicrobial activity of *Pseudomonas* sp. 4B to a bacteriocin from the supernatant. However, during the purification steps of this peptide, the activity was tested only against *Bacillus cereus* as indicator strain, not confirming if the same compound was responsible for inhibiting yeasts previously mentioned. Therefore, it is possible that the concentration of this compound in supernatant is very low. This fact can be compared with the results obtained by Sangmanee and Hongpattarakere

(2014) which tested the biological control of aflatoxigenic fungi using a *Lactobacillus plantarum* strain. Those authors found that the reduction of the fungal growth and aflatoxin production were achieved after increasing the supernatant concentration.

It is also possible that other compounds could be produced by *Pseudomonas* sp. 4B but the medium used for bacterial growth (BHI broth) may not be sufficient to synthesize the antifungal substance or higher concentrations. In assessing the antagonistic activity of *Pseudomonas* strains against phytopathogenic fungi, Cordero *et al.* (2012) found differences in the antifungal effect when used two different culture media. Hence, medium composition might be one of the factors that can influence in antagonistic activity. Additional research about different growth conditions is required to evaluate the potential of *Pseudomonas* sp. 4B in the production of antifungal metabolites.

The results in the present work suggest that *Pseudomonas* sp. 4B could inhibit filamentous fungi due to the ability to reduce the fungal growth parameters. The decrease in the levels of mycotoxins formed (AFB1 and OTA) produced by *A. flavus* A12 and *A. carbonarius* ITAL293, respectively, also indicates this bacterium as promising candidate for controlling the development of toxigenic fungi. Further experimentation will be necessary to elucidate the mode of action of the antagonistic bacterial strain in order to identify some antifungal compound responsible for this inhibition. This is the first reported study in which a *Pseudomonas* strain from aquatic environment was assessed about its potential antagonistic ability against toxigenic fungi. These finds encourages search for new biological control agents of toxigenic fungi, especially in the case fungi producers of mycotoxins.

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Conflict of Interest

The authors declare that no conflict of interest exists.

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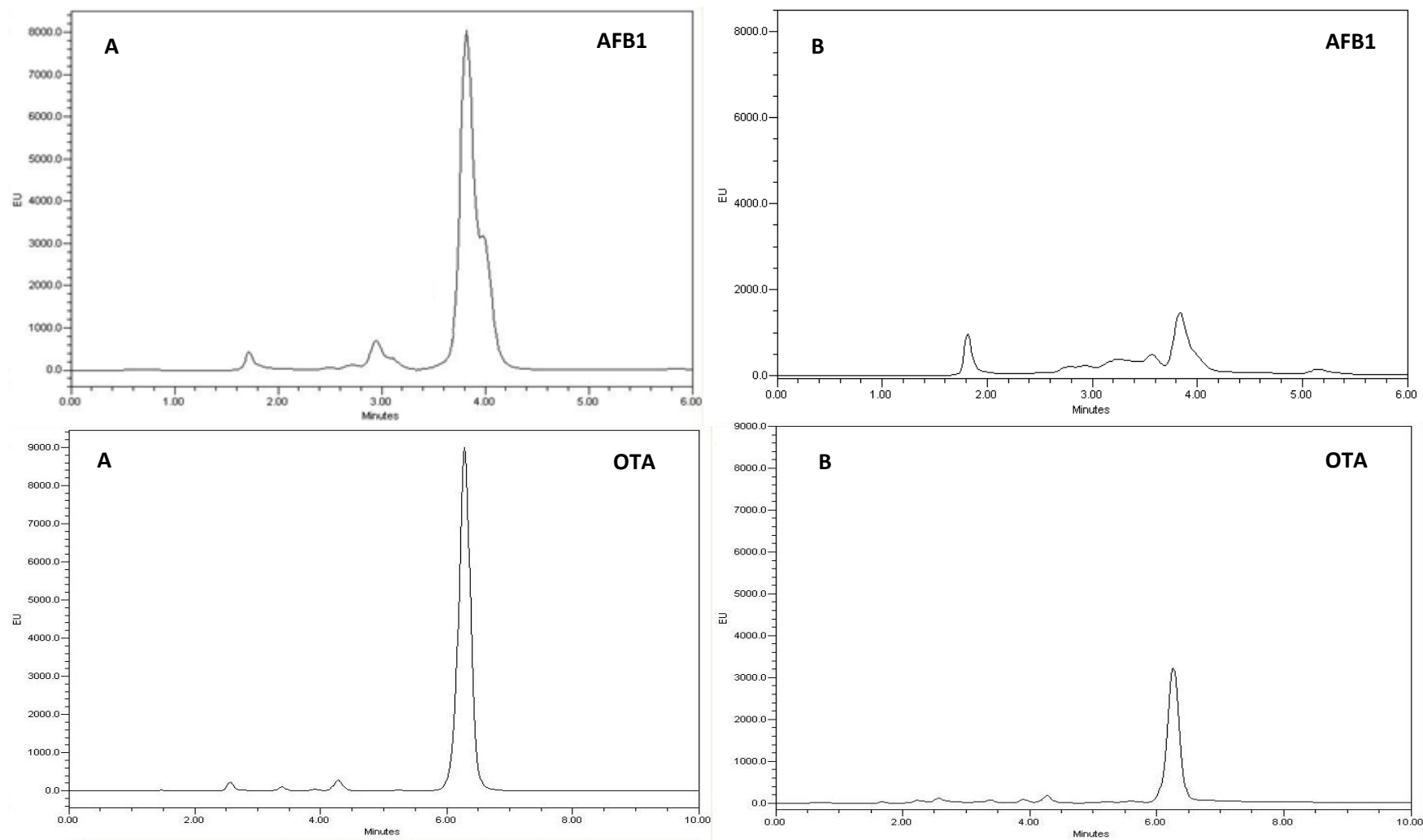
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Supporting Information

Figure S1. Profile of mycelial growth of *Aspergillus flavus* A12 (A), *Aspergillus parasiticus* 30BL (B), *Aspergillus carbonarius* ITAL293 (C), *Aspergillus* UCO2A sp. (D), *Monascus purpureus* NRRL1992 (E) and *Penicillium citrinum* ITAL197 (F) after 10 days of incubation at 25°C on PDA plates. The fungi were grown alone (■) or co-cultured with *Pseudomonas* sp. 4B (10^7 CFU ml⁻¹) (▲).

Figure S2. High performance liquid chromatographic analysis of *Pseudomonas* sp. 4B effect on aflatoxin B1 (AFB1) and ochratoxin A (OTA) production by *Aspergillus flavus* and *A. carbonarius*, respectively, after 10 days cultivation on PDA medium at 25°C. Mycotoxin production by fungal isolate cultivated alone (A) and with bacterial cells (B), peaks eluted at 3.9 and 6.5 min correspond to AFB1 and OTA, respectively.

Figure S2.



CAPÍTULO 4

Inhibition of filamentous fungi by ketoconazole-functionalized electrospun nanofibers

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Abstract

Nanotechnology strategies have been used for delivery and controlled release of antimicrobial drugs. Electrospun nanofibers can be versatile vehicles to incorporate antimicrobials. In this work, poly- ϵ -caprolactone nanofibers functionalized with ketoconazole were produced by electrospinning and tested against filamentous fungi. Ketoconazole-free nanofibers were produced as controls. Functionalized nanofibers showed antifungal activity against *Aspergillus flavus*, *A. carbonarius*, *A. niger*, *Aspergillus* sp. A29, *Fusarium oxysporum* and *Penicillium citrinum* by agar diffusion test. Inhibitory zones ranging from 6 to 44 mm were observed, this larger inhibition was against *A. flavus*. The nanofibers were incubated in different simulant solutions to evaluate the ketoconazole release, which was only detected in the solution containing 5% (v/v) Tween 20. Electron microscopy images showed the nanofibers with ketoconazole presented mean diameters of 526 nm, and the degradation of the nanofiber structures could be observed by electron microscopy after incubation in simulant solution. Infrared and thermal analyses indicated that ketoconazole was dispersed without chemical interactions with the polycaprolactone matrix. These results suggest that polycaprolactone nanofibers incorporating ketoconazole may be an interesting alternative to control pathogenic fungi.

Keywords: Antifungal; Electrospinning; Electrospray; Mycotoxin; Nanofibers

1. Introduction

Fungal infections are a serious public health problem, since they contribute substantially to human morbidity and mortality (Brown et al., 2012). Although most fungi are not dangerous, some strains can be harmful to health and fungi that are common in the environment can cause several fungal diseases (Hawksworth, 2001). Common filamentous fungi can represent a particular hazard to patients who are immunocompromised due to serious illnesses or undergoing immunosuppressive therapies (Brown et al., 2012). In addition, fungi that are normally found in air, soil or aquatic environments are likely associated with infections of skin wounds (Bartesteanu et al., 2014).

Azole antifungal drugs such as miconazole, econazole, oxiconazole, clotrimazole and ketoconazole are exceptionally water-insoluble, and therefore it is very difficult to administer and deliver these compounds to the infected site (Gupta and Cooper, 2008). Ketoconazole (Fig. 1A) is an antifungal agent with topical and systemic action that can be incorporated into several pharmaceutical forms. This triazole antifungal agent is used in the treatment of superficial and systemic fungal infections and for the treatment of seborrheic dermatitis. Ketoconazole has a high permeability, but low solubility in aqueous media, which is insufficient for the whole dose to be dissolved in the gastro-intestinal fluids under normal conditions (Van den Mooter et al., 2001). Some recent studies indicate that nanoparticles can be useful for improvement of ketoconazole delivery and bioavailability (Modi et al., 2013; Kakkar et al., 2015).

Nanotechnology has been used as an interesting tool for drug delivery and controlled release. A promise of nanostructured drug delivery is to reduce the drug amount and promote a more effective action, thus reducing the impact on human and animal health and the environment (Brandelli, 2015). Antimicrobial substances can be incorporated into several nanostructures, including nanospheres, nanovesicles, nanofibers and nanotubes (Brandelli, 2012; Brandelli and Taylor, 2015). Nanofibers have been mostly evaluated for their potential as wound dressings or scaffold for tissue engineering. However, nanofibers functionalized with antimicrobial agents can be a relevant alternative to protect against microbial pathogens (Heunis and Dicks, 2010; Wei and Wei, 2012). Among the processes used to nanofiber production, the electrospinning technique has been mostly used by its versatility, allowing the

incorporation of antimicrobials and other bioactive molecules (Damasceno et al., 2013; Oliveira et al., 2014).

The capability to produce nanofiber-based thin films functionalized with antimicrobials has a huge potential for applications in fungal control. However, the incorporation of ketoconazole into nanofibers has not been reported. In this study, ketoconazole was incorporated in poly- ϵ -caprolactone (PCL, Fig. 1B) nanofibers and this nanostructure was characterized and evaluated for its capability of inhibiting pathogenic fungi.

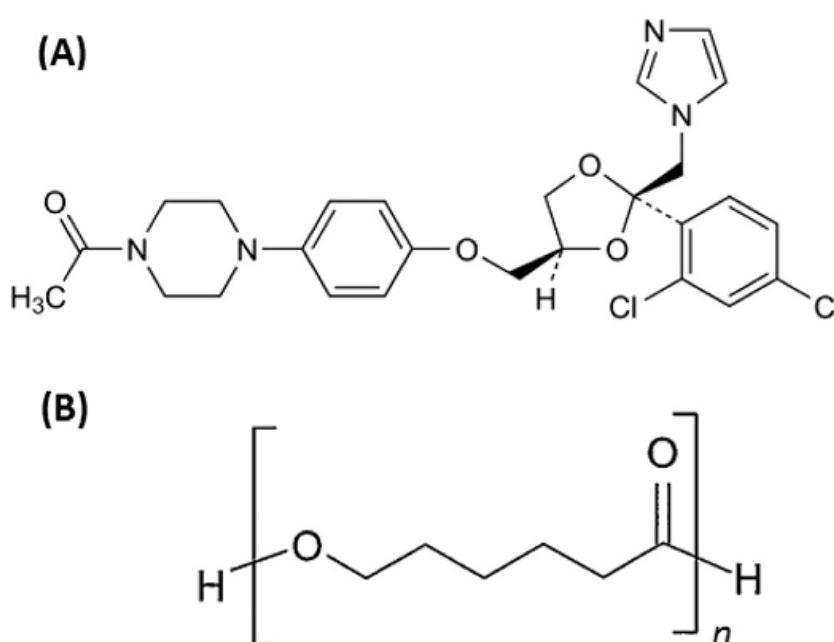


Fig. 1. Chemical structures of (A) ketoconazole and (B) poly- ϵ -caprolactone.

2. Materials and methods

2.1. Chemicals

Poly- ϵ -caprolactone (PCL; average MW 80,000) was obtained from Sigma Aldrich (St. Louis, MO, USA). Pharmaceutical grade ketoconazole was purchased from Piramal Healthcare (Maharashtra, India). Tetrahydrofuran (THF), chloroform (CHCl_3) and HPLC grade acetonitrile were from Merck (Darmstadt, Germany). All solutions were prepared using ultrapure water obtained from a Milli Q device (Millipore, Billerica, MA, USA).

2.2. Microorganisms

Aspergillus carbonarius and *Penicillium citrinum* strains were kindly provided by Instituto de Tecnologia de Alimentos (ITAL, Campinas, Brazil). *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus* sp. A29 and *Fusarium oxysporum* strains were from the collection of the Laboratório de Toxicologia de Alimentos, Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil). The fungal cultures were maintained on potato dextrose agar (PDA; Merck, Darmstadt, Germany) slants at 4 °C.

2.3. Preparation of PCL/ketoconazole nanofibers by electrospinning

Electrospinning was carried out essentially as described previously (Castañeda et al., 2014). PCL (100 g/l) was dissolved in a solution of THF:CHCl₃ (3:1) containing 1 g/l ketoconazole. PCL solution without the antifungal was prepared as control. The electrospinning was carried out under the following conditions: voltage of 30 kV, feeding rate of 0.05 ml/min; needle of 0.5 mm inner diameter; distance to the collector 16 cm. The nanofibers were collected on an aluminum plate (15 × 15 cm). The process was developed at 25 °C.

2.4. Fungal inhibition assays

For antifungal activity tests, the fungal strains were cultured on PDA slopes for 7 days at 25 °C. A solution containing 0.05% (v/v) Tween 80 was poured on each of the colonies and the spores harvested with the aid of a Drigalski loop. The suspension was collected and transferred to a sterile tube. The concentration of spore suspension was determined with a Neubauer chamber and adjusted with sterile distilled water to 1×10^6 spores/ml. One milliliter of this suspension was added to 99 ml of sterile PDA to 45 °C homogenized and transferred to a plate. After solidification of the media, nanofibers were cut and placed on agar plates. A disk containing 20 µg ketoconazole was used as positive control. Zone diameters were measured after five days of incubation at 25 ± 2 °C.

2.5. Ketoconazole quantification

The quantification of ketoconazole was performed by high performance liquid chromatography (HPLC) essentially as described elsewhere (Jat et al., 2012), using a Shimadzu Prominence system equipped with a diode array detector (Shimadzu, Tokyo, Japan). Analyses were performed using a Shim-pack HR-ODS column (250 × 3.0 mm, 3µm; Shimadzu). A 20µl sample was injected onto the HPLC by an auto sampler at 25 ± 1 °C. The column was eluted with an isocratic mobile phase prepared with ultrapure water: acetonitrile: 10 mM phosphate buffer pH 6.8 (51:45:4 ratio), with a flow rate of 1 ml/min. The column temperature was adjusted to 25 ± 1 °C and the detection wavelength was 238 nm. Mobile phase was filtered through 0.45 µm nylon filters and degassed by sonication prior to use. A standard calibration curve was prepared in the mobile phase with 1, 2, 5, 10, 25, 50 µg/ml ketoconazole. Regression analysis for HPLC provided a linear relationship of concentration to absorbance to over this range with a R² value of 0.9987. The lower limit of detection under these conditions was determined to be approximately 0.1 µg/ml.

2.6. Drug load

Drug load was determined using three different samples of the nanofibers. Samples of 4 mg were added to a solution of 0.2ml ultrapure water and 1.8 ml acetonitrile. After 30 min at 37 °C an aliquot was removed and the ketoconazole concentration was determined by HPLC. The drug load was calculated as DL = (released drug amount/sample weight).

2.7. Drug release assay

In order to describe the release of ketoconazole from the nanofibers, the migration assay was performed using different simulant solutions (Simoneau, 2009; Anvisa, 2010). These included distilled water (simulant A), 5% (v/v) Tween 20 (simulant B), 3% (v/v) acetic acid (simulant C), 15 and 50% (v/v) ethanol (simulants D and E, respectively). An amount of 10 mg of nanofiber sample was immersed into 5 ml of each simulant solution and shaken at 125 rpm at 30 °C. After 4, 24, 48, and 72 h, aliquots were harvested for quantification of ketoconazole concentration by HPLC and for determination of antifungal activity onto PDA agar plates as described above. A

recovery experiment was carried out by incubating standard ketoconazole into simulant solutions under the same conditions.

2.8. Scanning electron microscopy

The surface morphology of nanofibers was observed by scanning electron microscopy (SEM) using a JEOL JSM 5800 microscope (Jeol, Tokyo, Japan) at an accelerating voltage of 10 kV. Samples were sputtered with 5 nm Au/Pd prior to SEM imaging. The average fiber diameter (AFD) was determined from the SEM images, and around 100 fibers were analyzed for each treatment (Canbolat et al., 2014).

2.9. Thermal analysis

A thermogravimetric analyzer model TGA Discovery (TA Instruments, New Castle, DE, USA) was used for the thermal stability evaluation of PCL nanofibers. The samples were heated in platinum pans from 25 to 800 °C at the rate 10 °C min⁻¹ under nitrogen atmosphere (flow rate 25 ml min⁻¹). Differential scanning calorimetry (DSC) studies were performed using a DSC Q2000 apparatus (TA Instruments).

Samples equivalent to approximately 11 mg were placed in aluminum pans and heated from -80 to 200 °C with a heating rate of 20 °C/min under nitrogen atmosphere. An empty pan sealed with a cover pan was used as a reference sample (Ji et al., 2014). Crystallinity for PCL nanofibers was calculated as $\chi_c = \Delta H_m / \Delta H^{\circ}_c$, considering that the melting enthalpy of 100% crystalline PCL is 81.6 J/g (Danesin et al., 2012).

2.10. Infrared spectroscopy

Fourier transform infrared (FTIR) spectra were measured using a FTIR Varian 640-IR spectrometer (Varian Inc., Palo Alto, CA, USA) in attenuated total reflectance (ATR) mode with a diamond crystal. The scans were collected between 600 and 4000 cm⁻¹ at a 4 cm⁻¹ resolution. Near infrared (NIR) spectra were obtained using a Spectrum 100N spectrometer equipped with an ATR device (Perkin Elmer, Waltham, MA, USA). The samples were scanned from 4000 to 10,000 cm⁻¹ at a 4 cm⁻¹ resolution.

3. Results

3.1. Antimicrobial activity

The PCL nanofibers were initially tested against the pathogenic fungi *P. citrinum*, *A. carbonarius* and *A. flavus*. Control nanofibers caused no inhibition of fungal growth, while ketoconazole-functionalized nanofibers consistently inhibited all the tested fungi (Fig. S1). The inhibitory zones were significantly higher against *A. flavus* and *P. citrinum* as compared to those observed for *A. carbonarius*. Similar result was observed for pure ketoconazole, used as a positive control (Table 1).

Table 1

Inhibition of fungal growth by nanofibers containing ketoconazole.

Fungi	Diameter of inhibitory halo (mm)		
	Ketoconazole-functionalized nanofiber	Control nanofiber	Pure ketoconazole
<i>Penicillium citrinum</i>	20.0±5.0 ^a	0.0	16.7±0.6 ^a
<i>Aspergillus carbonarius</i>	6.3±1.0 ^b	0.0	8.0±1.0 ^b
<i>Aspergillus flavus</i>	44.4±10.0 ^c	0.0	16.3±0.6 ^a

^{a,b,c} Results are the means± s.e.m. of three independent experiments. Values followed by different superscript letters are significantly different ($P < 0.05$). Pure ketoconazole was used as positive control.

The drug load was determined using three different samples of ketoconazole-functionalized nanofibers, resulting in mean values of about $45.3 \pm 1.7 \mu\text{g}$ ketoconazole per mg of nanofiber.

The nanofibers were then subjected to drug release assays by incubation in five different solutions. Initially, ketoconazole recovery was tested using a final concentration of $25 \mu\text{g/ml}$. After 72 h, ketoconazole concentration in each solution was determined by HPLC. A substantial recovery was only observed in the saline solution containing 5% Tween 20 and 50% ethanol solution (Table 2). A second recovery experiment was conducted in solution B using $50 \mu\text{g/ml}$ ketoconazole, and after 72 h a mean value of $49.79 \mu\text{g/ml}$ was obtained, corresponding to 99.5% recovery.

Following this, ketoconazole-functionalized nanofibers were subjected to drug release assay using 10 mg samples disposed in 5 ml of each simulant solution. The amount of ketoconazole released after 3, 24, 48 and 72 h was determined and significant values were only observed for solution containing Tween 20 (Fig. 2).

Table 2

Ketoconazole recovery from simulating solutions.^a

Sample (simulant solution)	Concentration ($\mu\text{g/ml}$) ^b	Recovery (%)
A) Water	0	0
B) Saline +5% Tween 20	25.2	100.7
C) 20% Acetic acid	4.2	16.7
D) 10% Ethanol	18.1	72.5
E) 50% Ethanol	24.7	99.0

^a Ketoconazole was suspended to reach a final concentration of 25 $\mu\text{g/ml}$ in each solution.

^b Values were determined after 72 h incubation.

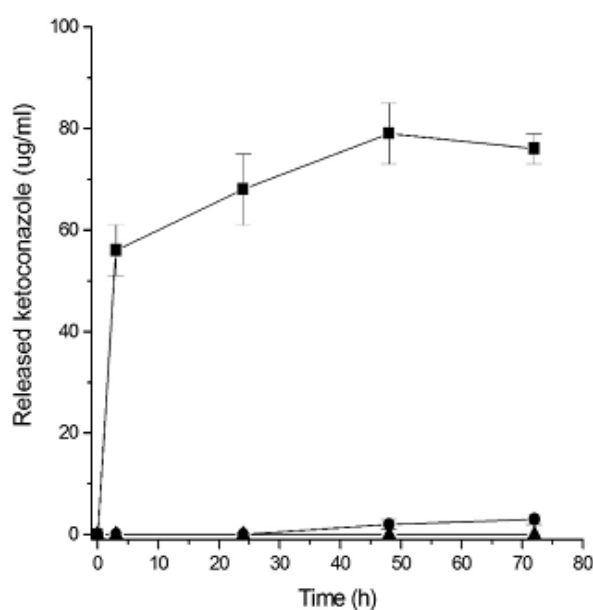


Fig. 2. Release of ketoconazole from polycaprolactone nanofibers during incubation in water (▲), 5% Tween 20 (■) or 3% acetic acid (●).

The aliquots removed from each incubation time were evaluated for their antifungal activity against several filamentous fungi. The results confirmed that only samples from simulant solution B, to which detectable amounts of ketoconazole was released, showed antifungal activity. Excepting for *F. oxysporum*, all strains tested were inhibited but no significant increase of inhibitory zones was observed after 24 h release (Table 3). The amount released within the first 3h was enough to inhibit the fungal strains tested, but the sustained release seems to maintain inhibition to at least 72 h. The inhibition diameters observed for the positive control of pure ketoconazole were higher against *A. flavus* and *P. citrinum*. Inhibition of fungal growth was not observed for samples from other simulant solutions (data not shown).

Table 3

Antifungal activity of ketoconazole functionalized-nanofibers.^a

Fungi	Time (h)				Pure ketoconazole
	3	24	48	72	
<i>A. flavus</i>	15.7±1.1	16.0±2.1	18.2±3.5	16.6±3.8	21.7±2.1
<i>Aspergillus</i> sp. A29	13.7±1.4	15.0±0.8	15.0±2.2	14.2±1.2	22.0±2.6
<i>A. niger</i>	5.7±0.9	9.0±1.4	7.8±0.1	7.7±2.0	9.0±0
<i>P. citrinum</i>	4.3±0.4	7.5±0.5	8.5±0.7	7.2±1.5	15.3±0.7
<i>F. oxysporum</i>	0.0	0.0	0.0	0.0	0.0
<i>A. carbonarius</i>	8.0±0.8	7.7±0.4	8.3±0.4	8.5±1.3	8.0±1.0

^a Results are the means ± s.e.m. of inhibition diameters (mm) obtained from three independent experiments. Pure ketoconazole was used as positive control.

3.2. Nanofiber characterization

The distribution charts of nanofiber diameter are depicted in Fig. 3. The control PCL nanofibers presented a mean diameter of 409 ± 142 nm, whereas the nanofibers functionalized with ketoconazole showed a mean diameter of 526 ± 148 nm. After 48 h incubation in simulant solution B, the mean diameter of control and functionalized nanofibers decreased to 278 ± 91 nm and 352 ± 97 nm, respectively (Fig. 3).

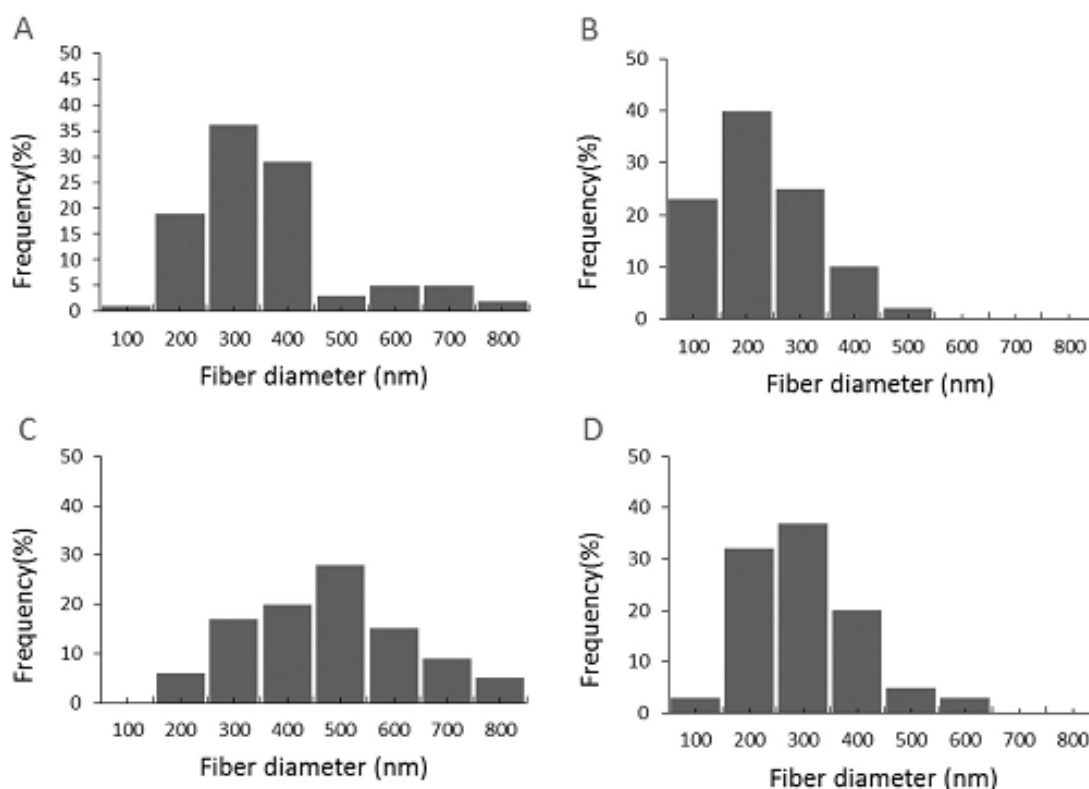


Fig. 3. Distribution of nanofiber diameter. Control nanofibers (A) before and (B) after 48 h incubation in saline solution containing 5% Tween 20. Nanofibers functionalized with ketoconazole (C) before and (D) after 48 h incubation in saline solution containing 5% Tween 20.

The nanofibers were observed by SEM and a typical string-like morphology was observed for control samples (Fig. 4A). When ketoconazole was included in the formulation, an increase in the fiber diameter was observed (Fig. 4C). After 48 h incubation in solution B, the control nanofibers were partially disrupted (Fig. 4B), while the ketoconazole-functionalized nanofibers were still intact, despite a clear decrease in the fiber diameter could be observed (Fig. 4D).

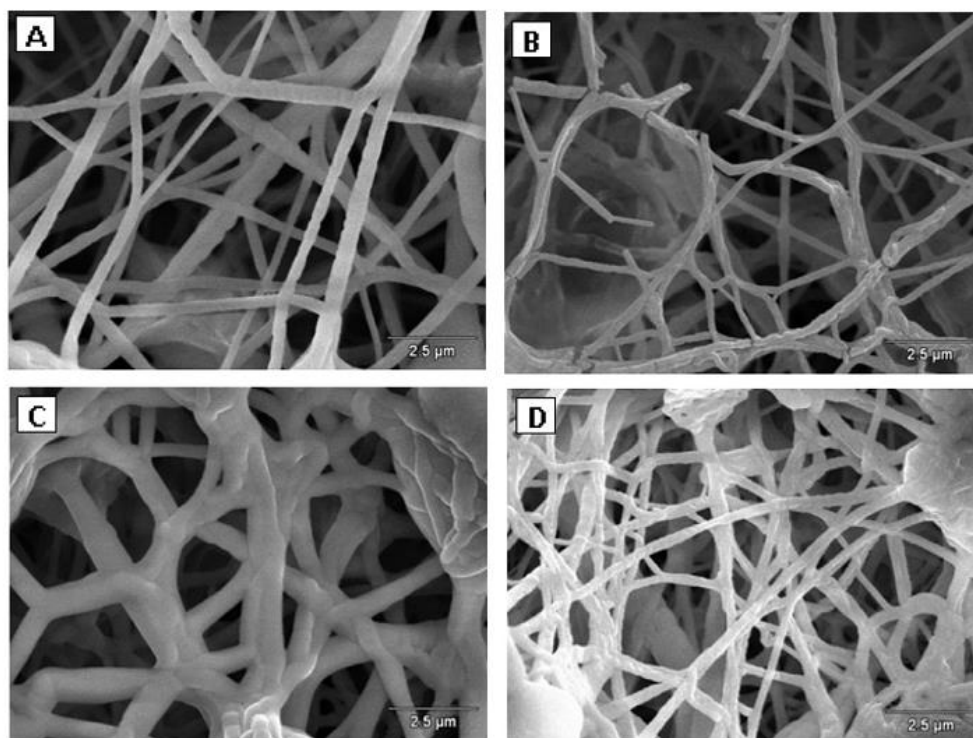


Fig. 4. Scanning electron microscopy of control and functionalized nanofibers. Control nanofibers (A) before and (B) after 48 h incubation in saline solution containing 5% Tween 20. Nanofibers functionalized with ketoconazole (C) before and (D) after 48 h incubation in saline solution containing 5% Tween 20.

Infrared spectroscopy studies were conducted to determine possible drug/polymer interactions. The results are presented in Fig. 5. The FTIR spectrum of pure ketoconazole displayed characteristic peaks of CO stretching vibration of carbonyl group, aromatic CC stretching, and C—O stretching of cyclic ether at 1645 cm^{-1} , 1505 cm^{-1} and 1240 cm^{-1} , respectively. Control and ketoconazole-functionalized nanofibers presented similar FTIR spectra, with the typical peaks of PCL at 2927 cm^{-1} (asymmetric CH_2 stretching), 2864 cm^{-1} (symmetric CH_2 stretching), 1727 cm^{-1} (carbonyl stretching), 1294 cm^{-1} (CO and C—C stretching), 1242 cm^{-1} (asymmetric C—O—C stretching) and 1173 cm^{-1} (symmetric C—O—C stretching) (Benkaddour et al., 2013). A peak at 1645 cm^{-1} , typical of ketoconazole, appeared in functionalized nanofiber but not in control nanofiber (Fig. 5).

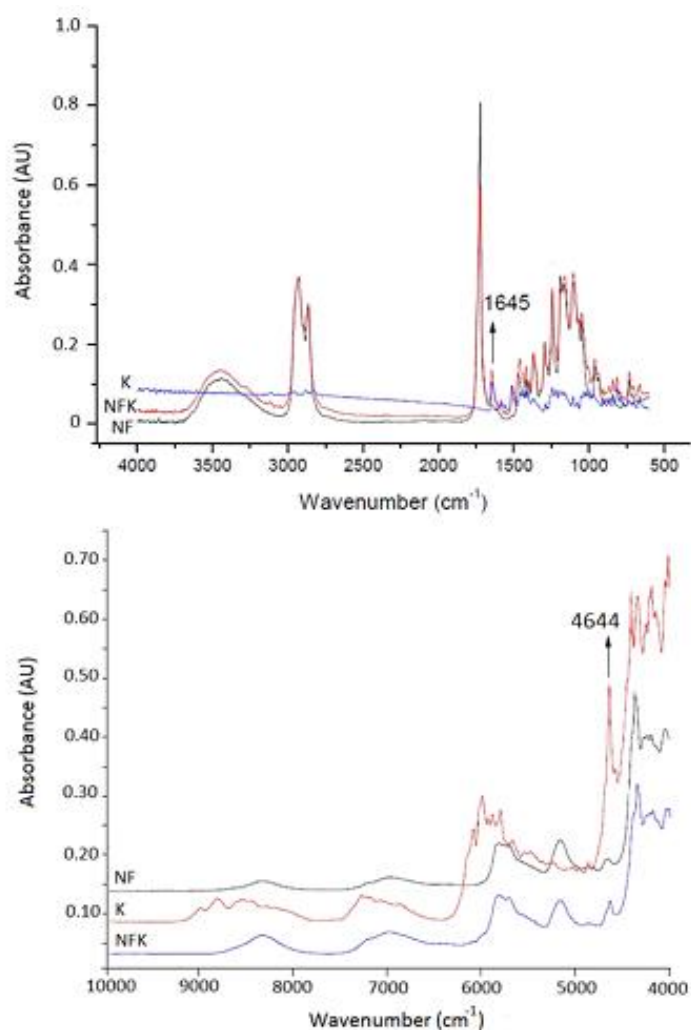


Fig. 5. Infrared spectroscopy analysis of nanofibers. Samples of control nanofibers (NF), ketoconazole-functionalized nanofibers (NFK) and pure ketoconazole were analyzed by FTIR (upper panel) and NIR (lower panel) using the attenuated total reflectance (ATR) methodology.

The NIR spectra of control and functionalized nanofibers showed absorbance at 5174 cm^{-1} (CO ester) and 5820 cm^{-1} (asymmetric CH_2 stretching), and broad bands at 6990 cm^{-1} and 8360 cm^{-1} assigned to C—H combination (Workman and Weyer, 2012). Despite that the NIR spectra were very similar, an increase in the sharp peak at 4644 cm^{-1} from ketoconazole was observed in functionalized nanofiber only (Fig. 5).

DSC studies were performed to understand the physical state of the drug in the electrospun nanofibers. The thermogram of crystalline ketoconazole showed a sharp endothermic fusion peak at $153\text{ }^\circ\text{C}$ (Fig. 6A), which is corresponding to the melting

point of ketoconazole (Marciniec et al., 2004). Also, an endothermic melting peak at 57 °C was observed for PCL nanofibers (Fig. 6A). The melting endotherm peak of ketoconazole was not detected in the functionalized nanofibers.

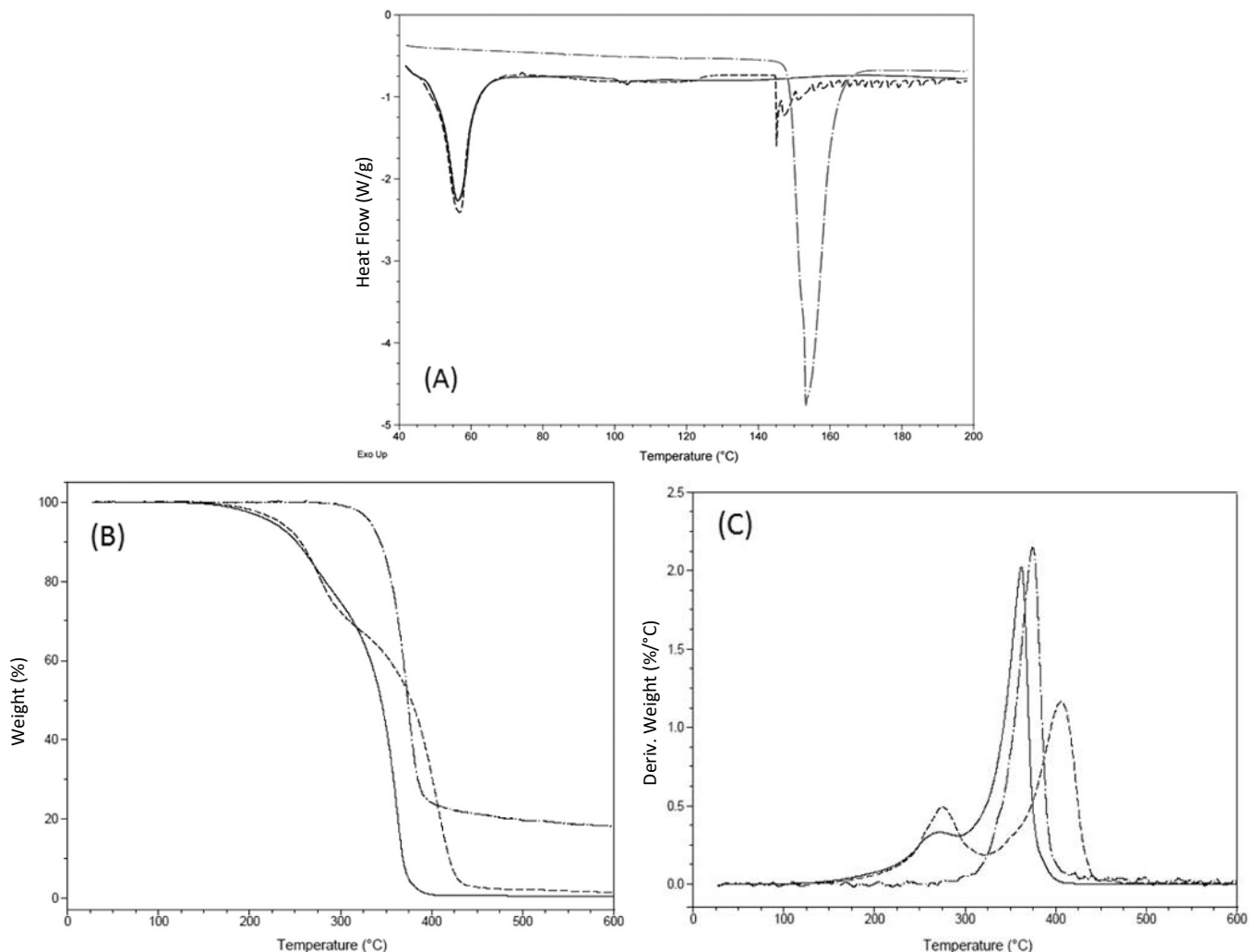


Fig. 6. Thermal analysis of nanofibers. Samples of control nanofibers (full lines), ketoconazole-functionalized nanofibers (dotted lines) and pure ketoconazole (point and dotted lines) were subjected to DSC (A) and TGA (B) analysis. Panel (C) illustrates the derivative TG analysis.

The values of glass transition temperature (T_g), onset of melting temperature (T_{onset}) and enthalpy of fusion (ΔH_m) were determined from DSC thermograms. Values for T_g , T_{onset} and ΔH_m were -46.7 °C, 50.5 °C and 33.4 J/g for control fibers, whereas values for ketoconazole-functionalized fibers were -48.5 °C, 50.6 °C and 36.2 J/g,

respectively. Crystallinity was calculated as 0.41 and 0.44 for control and functionalized PCL nanofibers, respectively.

TGA results showed that maximum thermal degradation of control nanofibers was at 275 - 380 °C, while for functionalized nanofiber it was at 275 - 425 °C (Fig. 6B). Weight losses below 250 °C were attributed to water loss while second weight loss at about 380 °C was attributed to decomposition of PCL polymer. In derivative thermogravimetric (DTG) analysis, both nanofibers exhibited two-peaks (Fig. 6C). Control nanofibers showed the maximum degradation rate at 360 °C while functionalized nanofibers at 405 °C.

4. Discussion

Electrospun nanofibers afford great flexibility for drug delivery applications, since their properties can be adapted by a cautious selection of polymers, drugs, solvents, emulsifying agents and electrospinning process conditions (Wei and Wei, 2012; Brandelli and Taylor, 2015). In this work, ketoconazole was incorporated in PCL nanofibers and the antimicrobial activity could be verified against filamentous fungi.

PCL has been used in drug delivery as a biodegradable polymer to form the hydrophobic core of polymeric nanoparticles, which are primarily used to carry and deliver poorly water-soluble drugs (Zhang et al., 2010). PCL nanospheres coated with poloxamer 188 has been used to encapsulate amphotericin B to combat *Candida albicans*, resulting in lower in vivo toxicity and reduced accumulation in kidneys and liver (Espuelas et al., 2003). Polyethyleneoxide (PEO) and PCL were used for the preparation of clotrimazole loaded nanofibrous patch. The resulting nanofibers showed in vitro drug release in simulated salivar fluid and antifungal activity, suggesting its therapeutic effectiveness in the treatment of oral candidiasis (Mir et al., 2014). Thus, the effective incorporation of ketoconazole into PCL nanofibers resulted in a functional nanostructure, indicating that electrospinning is a very promising nanotechnology-based approach to the formulation of such poorly water-soluble drugs. In addition, the solution containing Tween 20 was able to cause the largest amount of ketoconazole migration. Tween 20, a surfactant commonly used as emulsifier, can be capable to enhance the diffusion of ketoconazole from PCL matrix by increasing the drug apparent solubility in aqueous media.

The nanofibers obtained in this work showed a smooth morphology and average diameters ranging from 409 to 526 nm. In addition, no drug aggregates were observed on the surfaces of the electrospun nanofibers. These results were similar to those observed for PCL nanofibers containing metronidazole benzoate, although increasing the drug percentage caused the average diameter of electrospun nanofibers to decrease from 399 to 313 nm (Zamani et al., 2010). Clarithromycin was incorporated in poly-L-lactic acid (PLA) electrospun nanofibers, and the resulting nanocomposite presents an average diameter of 408 nm against 1091 nm for the control PLA nanofibers (Wei and Wei, 2012). Incorporation of the hydrophobic drug triclosan in PCL or PCL/PLA blends increased the diameter and modified surface morphology of the resulting electrospun fibers (Del Valle et al., 2011). The morphology and diameter of electrospun nanofibers are influenced by many parameters, including the molecular mass and concentration of the polymer and the viscosity, surface tension and conductivity of the solution. Low molecular mass solutions tends to for a “beads-on-string” morphology rather than regular fibers, while increased concentration of polymers induce alteration of spherical beads to rod-like, and lastly uniform fibers. Moreover, fibers with greater diameters are often obtained with the increase in the polymer concentration in solution (Brandelli and Taylor, 2015).

No relevant differences in FTIR or NIR spectra of control and functionalized nanofibers were observed, indicating no significant evidence of chemical interaction between drug and polymer, which confirms the stability of ketoconazole with its solid dispersion. FTIR analysis of clarithromycin-functionalized PLA nanofibers showed that the hydroxyl stretching peak of clarithromycin migrated from 3506 to 3462 cm^{-1} and the carbonyl stretching peak of PLA also shifted from 1756 to 1752 cm^{-1} in the nanocomposite, which indicate the formation of a hydrogen bond between the hydroxyl of clarithromycin and the carbonyl of PLA (Wei and Wei, 2012). No similar displacement of relevant peaks was observed for ketoconazole-functionalized PCL nanofibers, but the presence of typical peaks of ketoconazole at 1645 cm^{-1} in FTIR spectrum and 4644 cm^{-1} in NIR spectrum confirmed the incorporation of the drug in the nanostructure.

The thermal behavior of the PCL nanofibers was analyzed to investigate the physical state of the drug in the electrospun nanofibers. When guest molecules are incorporated in the nanofiber or in the crystal lattice, their melting point usually is shifted to a different temperature or disappear within the temperature range in which the PCL

matrix is decomposed (Zamani et al., 2010). The absence of detectable crystalline domain indicates a molecular dispersion of ketoconazole in amorphous state in the polymeric matrix. Similar results were reported to metronidazole-functionalized PCL nanofibers (Zamani et al., 2010), but thermal properties and crystallinity of PCL was affected by incorporation of triclosan, suggesting that the drug hinder the lamellar stacking of PCL (Del Valle et al., 2011). A decrease in the T_{onset} was observed for PCL nanofibers incorporating Lys-rich peptides (Danesin et al., 2012). In this work, by adding ketoconazole to the system, the melting enthalpy of PCL nanofibers was similar and appeared at almost the same temperature. These results show that adding ketoconazole in the semi-crystalline PCL nanofibers caused no relevant effect in the crystallinity.

In conclusion, it was demonstrated that PCL electrospun nanofibers are an interesting platform for delivery of ketoconazole, a poorly water-soluble antifungal drug. The inhibition of filamentous fungi indicates that this nanostructure could be effectively used to develop devices to be used as wound dressing or other strategies for combating fungal infections.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ejps.2016.01.014>.

Conflict of interests

None declared.

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Supplementary Figure S1

Veras et al., Inhibition of filamentous fungi by ketoconazole-functionalized electrospun nanofibers.

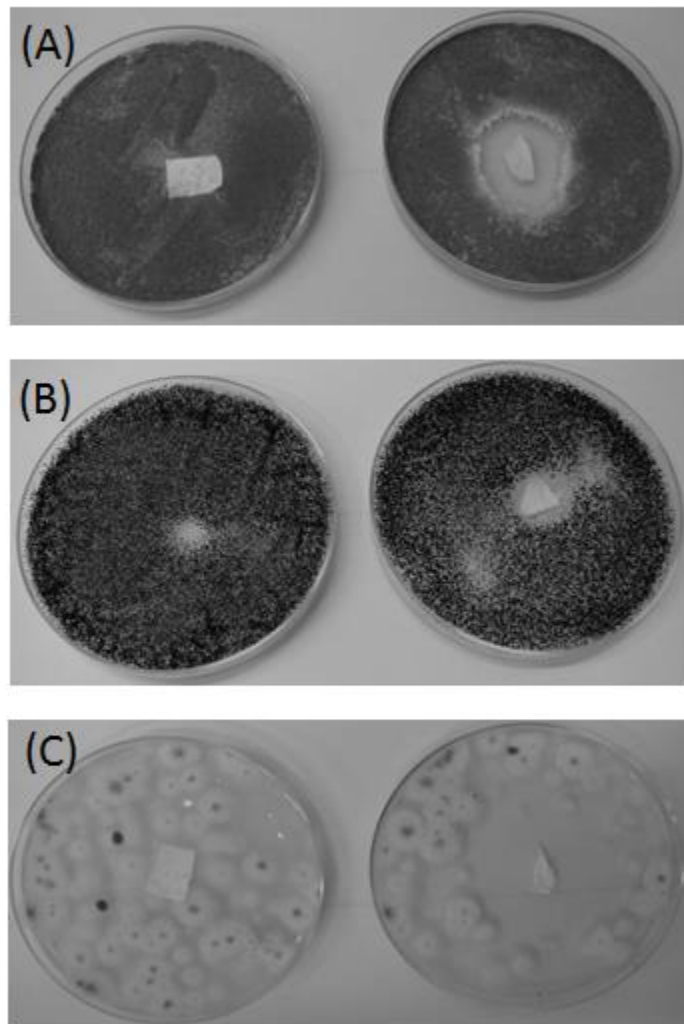


Fig. S1. Antifungal activity of ketoconazole-functionalized nanofibers. Left plates shown the control nanofibers and right plates correspond to nanofibers functionalized with ketoconazole. (A) *Penicillium citrinum*, (B) *Aspergillus carbonarius* and (C) *Aspergillus flavus*.

CAPÍTULO 5

Controle de fungos toxigênicos utilizando nanofibras de policaprolactona funcionalizadas com natamicina

Resumo

Nanofibras de poli- ϵ -caprolactona (PCL) foram produzidas pela técnica de eletrofiação e incorporadas com o antifúngico natamicina. Análises de microscopia eletrônica de varredura, espectroscopia de infravermelho e atividade antifúngica foram realizadas. Os fungos toxigênicos *Aspergillus flavus* A12, *A. carbonarius* ITAL 293 e *Penicillium citrinum* ITAL 197 foram utilizados como indicadores. Testes de liberação de natamicina a partir das nanofibras também foram realizados pelo contato com diferentes soluções simulantes. Nanofibras sem a incorporação do antifúngico foram utilizadas como controle. As análises microscópicas demonstraram que as nanofibras contendo natamicina apresentaram diâmetro médio maior do que as fibras controle (573 e 353 nm, respectivamente). Picos de absorção típicos de PCL foram observados em ambas as nanofibras após as análises espectroscópicas, indicando ausência de interações significativas entre polímero e antifúngico. A atividade antifúngica das nanofibras foi confirmada pela formação de halos de inibição de aproximadamente 13,5 mm em *A. carbonarius* ITAL 293 e *P. citrinum* ITAL 197. O contato das nanofibras com as soluções simulantes exibiu um perfil de liberação gradual da natamicina, com acumulação máxima do antifúngico de 44,4 $\mu\text{g/ml}$ na solução de Tween 20 (5%). Os resultados sugerem que nanofibras de poli- ϵ -caprolactona incorporadas com natamicina podem ser promissoras no controle de fungos toxigênicos.

Palavras-chave: nanotecnologia, nanomateriais, eletrofiação, antifúngicos, liberação controlada

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1. Introdução

A produção de nanofibras poliméricas vem demonstrando potencial de utilização em diversas áreas de aplicação, tais como a fabricação de filtros, sistemas de entrega de medicamentos, materiais médicos e até mesmo na área de alimentos [1, 2]. Entre as técnicas de fabricação de nanofibras, o processo denominado de eletrofiação, no qual envolve a aplicação de um campo elétrico, se destaca devido a sua simplicidade de operação, custo relativamente baixo e potencial para produção de membranas de nanofibras em escala industrial [1, 3]. De modo geral, o processo de eletrofiação produz nanofibras com características excepcionais, como por exemplo, grande área de superfície em relação ao volume, baixa densidade, alta porosidade e tamanho dos poros bastante reduzido [2]. Além disso, tal método permite a incorporação de uma variedade de compostos, incluindo fármacos e compostos bioativos, na superfície ou interior destas estruturas, proporcionando posterior liberação controlada das substâncias presentes e, portanto, garantindo determinada funcionalidade [1, 4, 5].

Diferentes agentes antimicrobianos também vêm sendo investigados quanto a sua eficácia de incorporação em nanofibras [3, 5]. Desta forma, nanofibras com funcionalidade antimicrobiana podem representar uma estratégia promissora para controlar a presença de microrganismos indesejáveis nos sistemas alimentares durante o armazenamento, transporte e manipulação de alguns produtos [2, 4]. Levando isso em consideração, a prevenção da contaminação fúngica em alimentos merece bastante atenção devido à capacidade de deterioração dos produtos e possibilidade de certos fungos em produzir micotoxinas [6]. Tais compostos apresentam riscos à saúde devido às propriedades carcinogênicas, imunossupressoras, mutagênicas, teratogênicas, além de elevada toxicidade [7]. Assim, o controle da presença de micotoxinas em alimentos também inclui a utilização de compostos que previnam o crescimento fúngico [6].

A natamicina, por exemplo, é um antifúngico polieno que vem sendo empregado como conservante alimentar. Esse agente se destaca pelo amplo espectro de ação e baixa toxicidade, além de não alterar as características organolépticas dos alimentos [8, 9]. No entanto, sua aplicação em determinados produtos pode não ser eficaz devido à pouca solubilidade em água [10]. Enquanto o polímero sintético conhecido como poli- ϵ -caprolactona (PCL), tem sido amplamente utilizado em diferentes aplicações, inclusive na área de alimentos [11–13]. Propriedades de

liberação controlada, capacidade de incorporação de vários compostos, hidrofobicidade e produção relativamente barata, em comparação com outros poliésteres alifáticos, são as principais características descritas para esse polímero biodegradável [12]. Deste modo, natamicina e PCL são potenciais candidatos a serem investigados como componentes de um sistema de liberação antimicrobiano com possibilidade para o desenvolvimento de embalagens.

Até o momento, a incorporação de natamicina em nanofibras de PCL, a caracterização dessas estruturas funcionalizadas e seu efeito *in vitro* contra fungos toxigênicos ainda não foram estudados. Neste artigo, nanofibras de PCL incorporadas com natamicina foram desenvolvidas e posteriormente avaliadas quanto ao seu potencial de utilização como material antifúngico. Especificamente, as características morfológicas e químicas, o perfil de liberação de natamicina e a atividade antifúngica dessas estruturas foram determinados.

2. Materiais e métodos

2.1 Reagentes

O polímero poli- ϵ -caprolactona (PCL; peso molecular 80.000 Da) foi adquirido da Sigma-Aldrich (St. Louis, EUA). Natamicina comercial em excipiente (50 % natamicina, 42 % lactose, 8 % umidade) foi adquirida da Granotec/Granolab (Paraná, Brasil) para a incorporação nas nanofibras, e um padrão do mesmo antifúngico (Sigma, EUA) com pureza acima de 95 % também foi utilizado para análises comparativas e como padrão analítico. Os solventes diclorometano, dimetilformamida (DMF), acetonitrila e metanol grau HPLC foram adquiridos da Merck (Darmstadt, Alemanha). Todas as soluções foram preparadas utilizando água ultrapura obtida de purificador do tipo Milli Q (Millipore, EUA).

2.2 Microrganismos

As linhagens *Aspergillus carbonarius* ITAL 293 e *Penicillium citrinum* ITAL 197 foram gentilmente fornecidas pelo Instituto de Tecnologia de Alimentos (ITAL, Campinas, Brasil). Estes isolados são produtores de ocratoxina A e citrinina, respectivamente. O isolado *Aspergillus flavus* A12 (produtor de aflatoxina B1) foi obtido do banco de culturas fúngicas do Laboratório de Toxicologia de Alimentos, Universidade Federal do Rio Grande do Sul (Porto Alegre, Brasil). Os isolados fúngicos foram mantidos a 4

°C em tubos inclinados contendo ágar batata dextrose (BDA; Merck, Darmstadt, Alemanha) e glicerol (20 %).

2.3 Preparação de Nanofibras por eletrofiação

O desenvolvimento de nanofibras através da técnica de eletrofiação foi realizado de acordo com o procedimento descrito por Castañeda, et al. [14]. Uma solução polimérica de PCL (100 g/l), contendo natamicina (0,1 %; m/v), foi preparada utilizando diclorometano:DMF (3:1). A solução foi colocada em uma seringa de 1 ml com agulha de diâmetro interno de 0,5 mm. A agulha foi posicionada a 16 cm de uma placa de alumínio (15 x 15 cm). A eletrofiação foi realizada aplicando uma voltagem de 30 kV nos eletrodos da agulha e placa coletora. O processo ocorreu sob taxa de alimentação (emissão do polímero) de 0,05 ml/min em temperatura controlada de 25 °C. Nanofibras sem a adição de natamicina na solução de PCL foram elaboradas como controle.

2.4 Caracterização das nanofibras

2.4.1 Análises microscópicas

A morfologia das nanofibras de PCL foi analisada por microscopia eletrônica de varredura (MEV). As amostras foram montadas em *stubs* e posteriormente metalizadas com ouro. A superfície das nanofibras foi observada em um microscópio eletrônico de varredura JEOL JSM 5800 (Jeol, Tóquio, Japão), o qual foi ajustado para uma voltagem de aceleração de 10 kV. A visualização das imagens foi feita sob faixa de magnificação de 10.000 x e o diâmetro médio das nanofibras foi determinado utilizando o programa ImageJ versão 1.45s (Instituto Nacional de Saúde, EUA) através da contagem de 100 fibras a partir de 3 imagens para cada amostra [15].

2.4.2 Espectroscopia de infravermelho

Análises de espectroscopia de infravermelho com transformada de Fourier (FTIR; *Fourier Transform Infrared*) foram realizadas utilizando espectrômetro FTIR Varian 640-IR (Varian Inc., Palo Alto, CA, EUA). Cada espectro foi obtido na região de 600 a 4000 cm^{-1} com resolução de 4 cm^{-1} . Nessas análises, a técnica de reflectância total atenuada (ATR) com utilização de cristal de diamante foi empregada.

Espectros de reflectância do infravermelho próximo (NIR; *Near Infrared Reflectance*) foram obtidos através de um analisador modelo Spectrum 100N (Perkin Elmer, Waltham, MA, EUA) equipado com dispositivo ATR. Os espectros foram gerados na faixa entre 4.000 e 10.000 cm^{-1} a uma resolução de 4 cm^{-1} .

2.5 Eficácia da incorporação de natamicina em nanofibras de PCL

2.5.1 Atividade antifúngica das nanofibras

Para o teste de inibição do crescimento fúngico, a metodologia empregada está descrita em Veras et al. [16] através da adição das nanofibras na superfície de placas de BDA contendo 1×10^6 esporos/ml para cada fungo avaliado. Discos contendo a mesma concentração de natamicina empregada para a fabricação de nanofibras funcionalizadas (0,1 %) foi usado como controle positivo.

2.5.2 Teste de liberação de natamicina

A capacidade de liberação do antifúngico presente nas nanofibras de PCL foi avaliada pelo teste de migração em diferentes soluções simulantes de alimentos [17, 18]. As soluções utilizadas foram: água destilada (simulante A), Tween 20 (5 %; v/v) (simulante B), ácido acético (3 %; v/v) (simulante C) e etanol (15 %; v/v) (simulante D). Para isso, amostras de nanofibras (20 mg) foram mantidas em contato com as soluções simulantes (20 ml) a 30 °C sob agitação de 125 rpm. Alíquotas foram coletadas nos tempos 0, 1, 2, 4, 8, 24, 48 e 72 horas para determinação da atividade antifúngica, conforme descrito no item anterior e quantificação da concentração de natamicina.

2.5.3 Carga antifúngica

A carga de natamicina incorporada nas nanofibras foi realizada pela adição de 200 μl de água ultrapura e 1,8 ml de acetonitrila em 4 mg da amostra. Após 30 minutos de incubação a 37 °C, uma alíquota foi retirada para determinação da concentração de natamicina. O valor da carga antifúngica foi calculado pela razão entre a concentração de natamicina liberada e o peso da amostra.

2.6 Quantificação da natamicina

A determinação de natamicina foi realizada por Cromatografia Líquida de Alta Eficiência (CLAE) utilizando um cromatógrafo (Waters, modelo E2695) com detector de arranjo de diodos (DAD 2998, Waters). A separação foi feita em coluna RP 18 (5 μ m, 4,6 x 150 mm) (Waters, Xbridge™ Shield) empregando eluição em gradiente de acordo com a Tabela 1. A fase móvel utilizada era composta de acetonitrila:ácido fosfórico (0,1 %; v/v). O volume de injeção da amostra foi de 10 μ l e o tempo de corrida de 15 minutos, com taxa de fluxo de 1,23 ml/min. A temperatura da coluna foi ajustada para 30 °C e o comprimento de onda do detector foi de 210 nm.

Tabela 1 Perfil do gradiente de eluição para determinação de natamicina por Cromatografia Líquida de Alta Eficiência (CLAE). Os eluentes utilizados foram acetonitrila (A) e água acidificada com 0,1 % de ácido fosfórico (B)

Tempo (minutos)	Eluentes		Fluxo (ml/min)
	A%	B%	
0	10	90	1,23
10	80	20	1,23
12	80	20	1,23
15	10	90	1,23

Um padrão de natamicina (Sigma, EUA) com pureza acima de 95 % foi dissolvido em metanol acidificado com 0,1 % de ácido trifluoracético (Sigma) para obter diferentes concentrações (1, 2, 5, 10, 25 e 50 μ g/ml). Estas soluções foram injetadas no cromatógrafo e uma curva padrão foi construída relacionando a concentração do padrão com a resposta no detector (área do pico cromatográfico). A equação de análise de regressão obtida apresentou um coeficiente de determinação (R^2) no valor de 0,9997. Os picos das amostras foram identificados pela comparação com o tempo de retenção do padrão de natamicina (5,6 minutos) e a quantificação foi realizada baseada na curva de calibração do padrão, expressando os resultados em mg/l. As condições de análise cromatográfica descritas acima foram realizadas de acordo com as recomendações do fabricante do padrão. Os valores de limite de detecção e limite de quantificação foram 0,01 e 0,04 μ g/ml, respectivamente.

2.7 Análises estatísticas

Os resultados obtidos foram comparados por meio de análise de variância (ANOVA) a um nível de significância de 5 %. Todos os valores são apresentados como médias de três determinações, e os resultados foram expressos como valores médios \pm desvio padrão (DP).

3. Resultados e discussão

Durante o processo de eletrofiação, nanofibras de PCL incorporadas com natamicina foram obtidas. As imagens de microscopia (Fig. 1) demonstram que as nanofibras apresentaram aspecto uniforme, com estruturas bem formadas, de superfície lisa e sem granulação. Também foi observado que a adição de natamicina influenciou no aumento do diâmetro das fibras produzidas. Nanofibras de PCL sem a incorporação de natamicina (tratamento controle) apresentaram diâmetro médio de 353 ± 119 nm. Por outro lado, aquelas que adicionaram o antifúngico em sua formulação exibiram diâmetros com valor médio de 573 ± 144 nm. Outras alterações morfológicas, ao comparar os dois tipos de nanofibras, não foram observadas. A Figura 1 ainda demonstra a ampla distribuição de diâmetro das nanoestruturas, variando de 112 a 997 nm.

O aumento no diâmetro de nanofibras após a adição de determinados compostos, incluindo antimicrobianos, em sua matriz polimérica tem sido comumente relatado [19–21]. Lakshminarayanan et al. [20] utilizaram a técnica de eletrofiação para o desenvolvimento de fibras de gelatina incorporadas com diferentes antifúngicos. Estes autores verificaram que, dentre os cinco compostos testados, a natamicina exerceu maior influência sobre o diâmetro das estruturas. Após sua adição na formulação da matriz, o diâmetro das fibras aumentou de 800 para 1800 nm. Os autores ainda verificaram que apenas um dos antifúngicos incorporados, o fluconazol, não afetou o diâmetro das fibras produzidas. Além disso, as características morfológicas das nanoestruturas no presente estudo foram semelhantes às aquelas obtidas durante o desenvolvimento de nanofibras de PCL funcionalizadas com o antifúngico cetoconazol [16], incluindo o aumento no diâmetro das fibras durante a incorporação do fármaco.

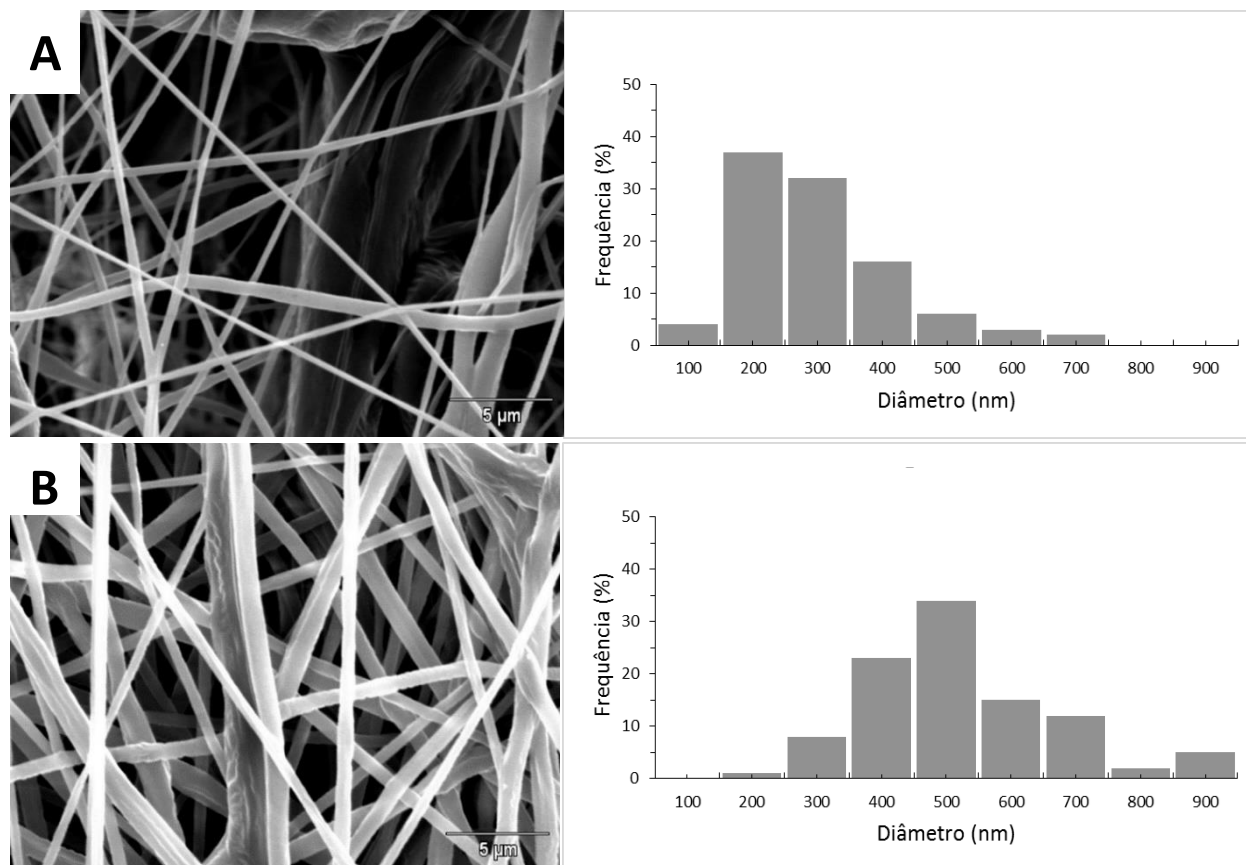


Fig. 1 Imagens de Microscopia Eletrônica de Varredura (aumento de 5.000 x) (esquerda) e Gráfico de distribuição do diâmetro (direita) de nanofibras de policaprolactona sem antifúngico (A) e nanofibras funcionalizadas com 0,1 % de natamicina (B)

Vários fatores podem interferir na morfologia e diâmetro de nanofibras obtidas por eletrofiação. Geralmente, a massa molecular e a concentração do polímero, além da viscosidade da solução são os que mais influenciam [2, 22]. Zahedi et al. [23] comentam, por exemplo, que soluções poliméricas com viscosidade elevada resultam em maior resistência no alongamento do jato polimérico injetado pela agulha durante o processo de eletrofiação ocasionando a formação de fibras mais espessas.

Quanto às análises de espectroscopia de infravermelho, os resultados de FTIR indicam que a presença de natamicina não altera o perfil dos espectros das nanofibras de PCL (Fig. 2A). Bandas de absorção referentes aos alongamentos assimétrico e simétrico de metileno (CH_2) foram observadas nas regiões de 2927 e 2864 cm^{-1} , respectivamente. Picos à 1727 cm^{-1} (estiramento do grupo carbonila), 1294 cm^{-1} (estiramento das ligações CO e $\text{C}-\text{C}$), 1242 cm^{-1} (estiramento assimétrico da ligação $\text{C}-\text{O}-\text{C}$) e 1173 cm^{-1} (estiramento simétrico de $\text{C}-\text{O}-\text{C}$) [24] também foram

visualizados. No entanto, a presença de picos de absorção entre 1270 e 1180 cm^{-1} também se refere à existência de diferentes grupos CO típicos da natamicina [25] e, portanto, poderia justificar o discreto aumento desses picos nos espectros das nanofibras incorporadas com o antifúngico.

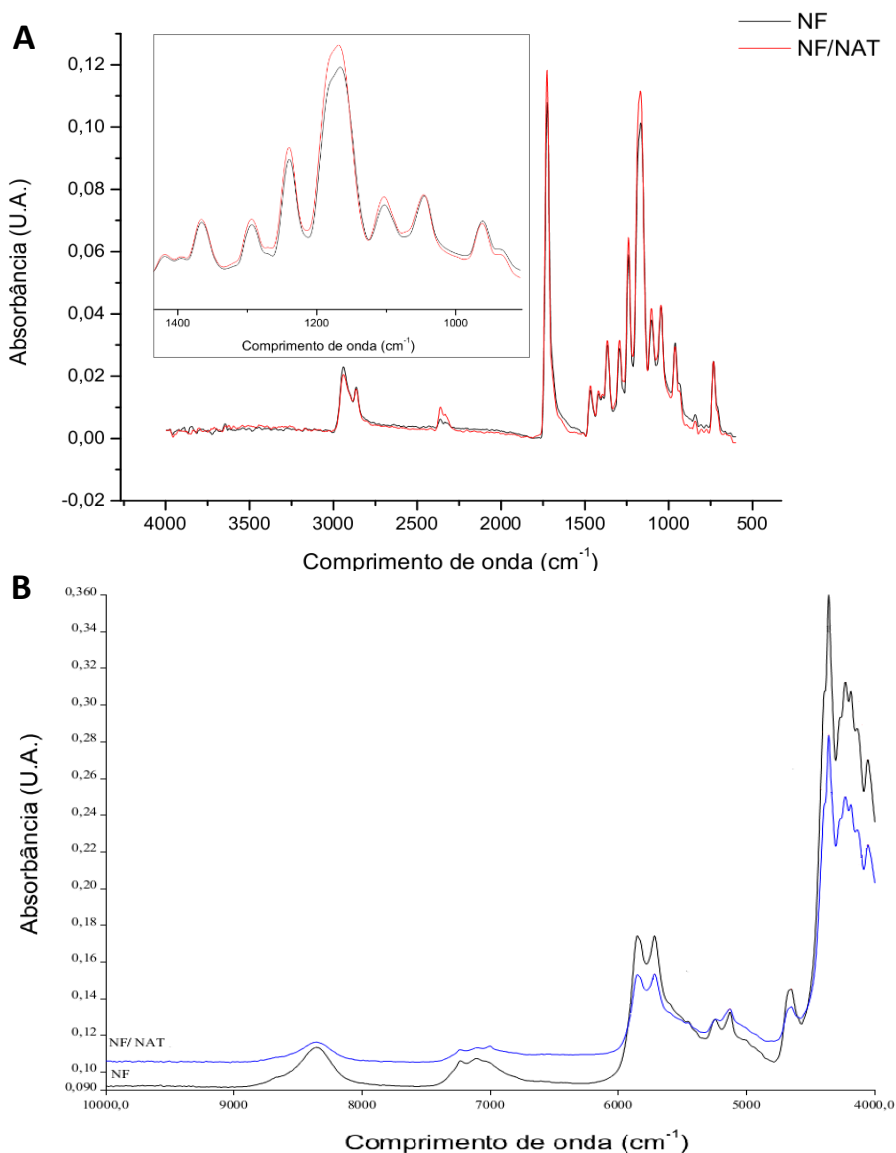


Fig. 2 Espectros de FTIR (A) e NIR (B) correspondentes às nanofibras de policaprolactona sem antifúngico (NF) e nanofibras incorporadas com 0,1 % de natamicina (NF/NAT). A figura ainda apresenta a ampliação dos espectros de FTIR entre 1400 e 1000 cm^{-1}

Através dos espectros no infravermelho médio (Fig. 2B) também pode-se observar que as nanofibras controle e funcionalizadas apresentaram picos semelhantes e típicos de PCL: bandas ao redor de 5820 cm^{-1} provenientes de

estiramentos assimétricos de metileno (CH_2), uma absorção que ocorre na região próxima a 5174 cm^{-1} (éster CO) e bandas largas de baixa intensidade na região de 6990 cm^{-1} a 8360 cm^{-1} correspondentes às vibrações de deformação axial da ligação C-H [26]. Veras et al. [16] comentam que a ausência de diferenças relevantes nos espectros de nanofibras de PCL quando incorporadas com cetoconazol pode indicar a falta de interação química significativa entre o antifúngico e o polímero. Este fato também poderia ser aplicado às nanofibras funcionalizadas com natamicina.

As propriedades antifúngicas das membranas de nanofibras de PCL foram avaliadas para determinar se a atividade da natamicina incorporada permanece após a eletrofiação. Foi possível verificar a presença de zonas de inibição do crescimento fúngico ao redor das membranas incorporadas com natamicina (Fig. 3). Os isolados *A. carbonarius* ITAL 293 e *P. citrinum* ITAL 197 foram sensíveis ao efeito das nanofibras funcionalizadas, apresentando halos de inibição próximos de 13,5 mm (Tabela 2). Estes resultados indicam que a atividade antifúngica da natamicina foi mantida durante o procedimento de eletrofiação. Como esperado, os discos contendo natamicina (controle positivo) demonstraram efeito fungicida contra todos os fungos, sendo possível visualizar halos de inibição variando de 10 a 17 mm. Por outro lado, nanofibras sem a incorporação de natamicina em sua matriz (controle negativo) não foram capazes de formar zonas de inibição fúngica, confirmando a ausência de qualquer efeito deste material sobre o crescimento dos fungos.

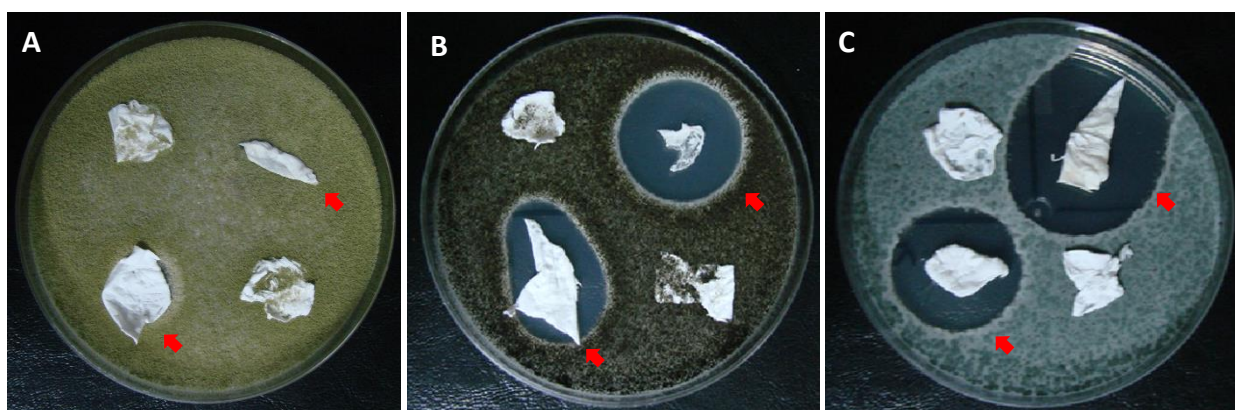


Fig. 3 Atividade antifúngica de nanofibras de poli- ϵ -caprolactona, obtidas pela técnica de eletrofiação, contra *Aspergillus flavus* A12 (A), *A. carbonarius* ITAL 293 (B) e *Penicillium citrinum* ITAL 197 (C). As setas correspondem à utilização de nanofibras incorporadas com 0,1% de natamicina

Tabela 2 Atividade antifúngica de nanofibras de poli- ϵ -caprolactona contra fungos toxigênicos cultivados a 25 °C durante 5 dias. Natamicina em discos de papel (0,1 %) foi utilizada como controle positivo

Fungos	Halo de inibição (mm)		
	NF/NAT	NF/C	Controle positivo
<i>Aspergillus flavus</i> A12	0 \pm 0b	0 \pm 0b	10,0 \pm 0,1a
<i>A. carbonarius</i> ITAL 293	13 \pm 3,68b	0 \pm 0c	17,0 \pm 1,0a
<i>Penicillium citrinum</i> ITAL 197	14 \pm 5,81a	0 \pm 0b	15,7 \pm 0,6a

Letras diferentes na mesma linha indicam diferença estatística pelo teste de Tukey ($P < 0,05$). Valores correspondem à média de 2 experimentos realizados em triplicatas; \pm , desvio padrão. NF/NAT, Nanofibras incorporadas com 0,1% de natamicina; NF/C, Nanofibras sem adição do antifúngico

A eficácia da incorporação de compostos com ação antifúngica em nanoestruturas tem sido comprovada em outros estudos [16, 22, 27]. Valores semelhantes de halos de inibição (abaixo de 15 mm) foram encontrados por Lakshminarayanan et al. [20] durante a adição de natamicina em nanofibras de gelatina. Estes autores também desenvolveram nanofibras funcionalizadas com itraconazol e fuclonazol, no entanto a incorporação destes antifúngicos não foi eficaz. Neste caso, as nanofibras contendo estes compostos não conseguiram inibir o crescimento da maioria dos fungos testados.

Quanto a ausência de inibição do crescimento de *A. flavus* A12, Fajardo et al. [28] comentam que o alto conteúdo de água em meios de cultura pode alterar as propriedades de liberação de natamicina em sistemas de distribuição controlada. Desta forma, a concentração deste antifúngico liberado no meio utilizado (BDA) pode não ter sido suficiente para inibir especificamente o crescimento deste fungo. No entanto, apesar de nenhuma zona de inibição fúngica ter sido formada pelas nanofibras, não houve crescimento do fungo sob a superfície da membrana funcionalizada. Tal fato não foi observado nas nanofibras controle. Esse mesmo comportamento foi verificado por Kim et al. [29] ao testar nanofibras de nylon contendo floxina B contra *A. fumigatus*.

Além da eficácia de incorporação da natamicina nas nanofibras de PCL, o perfil de liberação do antifúngico destas membranas foi avaliado durante 72 horas de contato com diferentes soluções simulantes. A partir da Figura 4 pôde-se observar que ocorreu liberação gradual de natamicina em todas as soluções simulantes

empregadas, embora com diferentes perfis. A maior concentração de natamicina liberada ocorreu em 48 horas de contato em solução de Tween 20 (simulante B), com valor correspondente a 44,4 $\mu\text{g/ml}$. O segundo melhor perfil de liberação foi observado em etanol 15 % (simulante D), porém a quantidade máxima de natamicina na solução foi de 17,1 $\mu\text{g/ml}$ (após 72 horas). O conteúdo total de natamicina acumulada em água destilada ou ácido acético após o final do experimento foi considerado muito baixo, com valores próximos a 5 $\mu\text{g/ml}$.

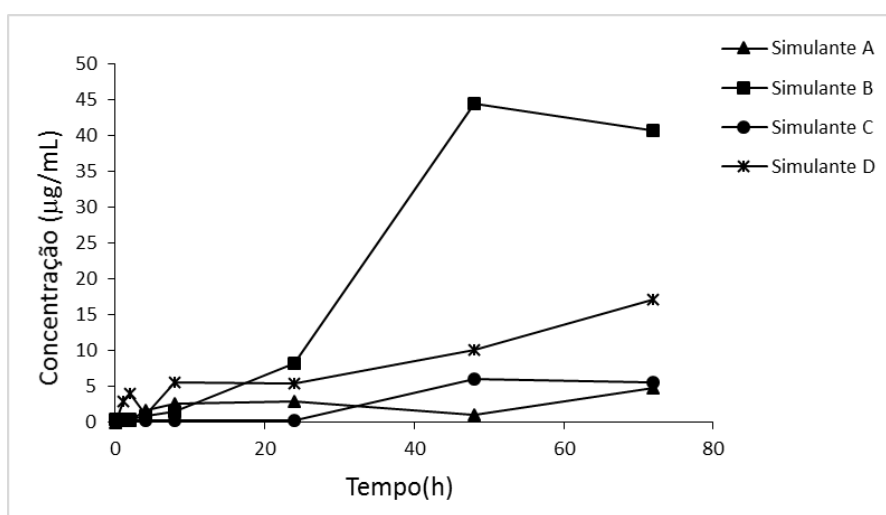


Fig. 4 Perfil de liberação de natamicina a partir de nanofibras de policaprolactona durante 72 horas de contato com diferentes soluções simulantes. Simulantes A, B, C e D correspondem à água destilada, Tween 20 (5 %), ácido acético (3 %) e etanol (15 %), respectivamente

Os resultados obtidos demonstram um efeito expressivo para um sistema de administração da natamicina uma vez que o composto deve ser liberado de forma gradual e, conseqüentemente, possuir um tempo de permanência prolongado. Resultados similares foram observados por Chandasana et al. [30] em nanopartículas de PCL incorporadas com natamicina. Neste caso, a liberação gradual em simulante de fluido lacrimal foi verificada após o contato de membranas de diálise contendo as nanopartículas funcionalizadas, porém com transferência total da carga de natamicina para o meio simulante em apenas 8 horas.

Após determinação da carga antifúngica das nanofibras de PCL (180,5 μg de natamicina por mg de nanofibra), foi possível verificar que a concentração acumulada na solução de Tween 20 corresponde a aproximadamente 24 % do conteúdo total. Alguns estudos avaliaram a liberação de compostos incorporados em nanoestruturas

durante um período maior que 72 horas, obtendo valores próximos a 100% da carga total somente após 5 dias de incubação do material [31–33]. Portanto, é provável que um maior tempo de contato das nanofibras de PCL com as soluções simulantes favoreça a liberação de uma maior concentração do antifúngico.

Também foi possível observar que as soluções de Tween 20 (5 %), ácido acético (3 %) e etanol (15 %) apresentaram atividade antifúngica ao final do experimento de liberação da natamicina. Halos de inibição durante o crescimento dos fungos em meio BDA, variando de 8 a 12 mm, foram visualizados após a aplicação de 20 µl das soluções simulantes (dados não mostrados). Estes resultados indicam que as propriedades antifúngicas da natamicina foram mantidas, sugerindo que os riscos de sua inativação durante o processo de liberação são mínimos.

Vários tipos de alimentos estão expostos à contaminação microbiana durante seu processo de fabricação, servindo como substrato para o crescimento de fungos toxigênicos e produção de suas micotoxinas [6]. Apesar da natamicina ser comumente utilizada como conservante, sua hidrofobicidade pode comprometer a eficácia da aplicação em alguns alimentos [8, 10]. Portanto, a capacidade das nanofibras de PCL em liberar natamicina nas soluções utilizadas como simulantes de diversos tipos de alimentos reflete na importância deste material para o controle do crescimento fúngico em determinados produtos alimentícios.

4. Conclusão

Neste estudo, o desenvolvimento de nanofibras de PCL incorporadas com natamicina foi obtido com êxito. A adição do antifúngico na matriz polimérica destas estruturas não causou alterações expressivas nos parâmetros investigados. Os resultados ainda demonstraram que a atividade antifúngica da natamicina foi mantida após o processo de eletrospinning uma vez que as nanofibras funcionalizadas inibiram o crescimento de fungos toxigênicos. Além disso, a liberação gradual do antifúngico presente nestas nanoestruturas, através do contato com diferentes soluções simulantes de alimentos, também foi observada. Diante disso, nanofibras de poli-ε-caprolactona funcionalizadas com natamicina apresentam potencial de utilização como material antifúngico, podendo contribuir para futuras pesquisas sobre o desenvolvimento de embalagens de alimentos com liberação controlada de compostos antimicrobianos.

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CAPÍTULO 6

Discussão geral

As estratégias de controle da exposição às micotoxinas incluem a prevenção do crescimento fúngico em todas as etapas da cadeia de produção de alimentos. A grande diversidade fúngica, as inúmeras possibilidades e condições de contaminação microbiana e as limitações de algumas práticas de controle exigem a busca por diferentes formas de combater o desenvolvimento de fungos (toxigênicos e/ou deteriorantes) em alimentos (ZAIN et al. 2011; TORRES et al., 2014). Esse estudo abordou duas estratégias para a prevenção do crescimento fúngico: o emprego de agentes de controle biológico e a utilização de nanomateriais incorporados com antifúngicos.

Linhagens bacterianas dos gêneros *Bacillus* e *Pseudomonas* foram investigadas como agentes de controle biológico. *Bacillus* spp. (provenientes da Região Amazônica) e *Pseudomonas* sp. 4B (isolada de um lago de tratamento de efluentes) apresentaram, pela primeira vez, resultados promissores no controle de fungos toxigênicos. Estes representantes foram capazes de reduzir em até 100% as taxas de crescimento micelial, a esporulação e a germinação de esporos para alguns fungos testados.

Uma vez que os gêneros *Bacillus* e *Pseudomonas* se destacam dentre outros agentes de controle biológico, principalmente pela capacidade de produzir vários compostos antimicrobianos (MAUGHAN; VAN DER AUWERA, 2011; QIAN et al., 2016), testes de identificação dos possíveis compostos envolvidos na atividade antifúngica foram realizados.

A habilidade de duas linhagens de *Bacillus* em produzir lipopeptídeos como a iturina A e isômeros de surfactina foi confirmada. Estes compostos são reconhecidos como metabólitos secundários produzidos por espécies de *Bacillus* que podem contribuir no controle de crescimento fúngico (ZHAO et al., 2010; YE et al., 2012; JIANG et al., 2014). No entanto, estudos recentes demonstraram que diferentes lipopeptídeos podem ser produzidos simultaneamente por *Bacillus* spp. (BENITEZ et al., 2010; AYED et al., 2015; INÈS; DHOUHA, 2015). É possível que as linhagens bacterianas investigadas produzam uma mistura de tais compostos, uma vez que, embora *Bacillus* sp. P1 tenha produzido as maiores concentrações de iturina A e

surfactinas, não apresentou o melhor perfil de atividade antifúngica quando comparado com as outras linhagens. Fengicina, micosubtilina, bacilomicina e kurstakina são exemplos de outros lipopeptídeos que podem ser produzidos pelo gênero *Bacillus* (THASANA et al., 2010; SLIVINSKI et al., 2012; INÈS; DHOUHA, 2015; PRETORIUS et al., 2015). Além disso, proteínas antifúngicas, incluindo enzimas, tais como quitinases, glucanases e proteases, são também sintetizadas por espécies de *Bacillus* (OSCÁRIZ; PISABARRO, 2000; MANJULA et al., 2004; THAKAEW; NIAMSUP, 2013; MNIF; GHRIBI, 2015). Desta forma, a produção de tais compostos pelos *Bacillus* utilizados neste estudo ainda não pode ser descartada. Portanto, pesquisas sobre a produção e identificação de outros metabólitos antifúngicos devem ser realizadas, além de estudos sobre a otimização e purificação dos lipopeptídeos produzidos por estas linhagens.

Este é o primeiro estudo sobre o controle de fungos toxigênicos utilizando representantes do gênero *Bacillus* isolados do intestino de peixes da Bacia Amazônica. Tal fato merece atenção uma vez que a maioria dos trabalhos investiga o potencial antifúngico de *Bacillus* provenientes de ambientes que estão associados à fitosfera (CHO et al., 2009; ZHAO et al. 2010; CALDEIRA et al., 2011; CAO et al., 2011b; KRID et al., 2012; AFSHARMANESH et al., 2014; GONG et al., 2014; LI et al., 2014; BEN KHEDHER et al., 2015; YANG et al., 2015). Torres et al. (2016) verificaram o potencial antifúngico de *B. subtilis* isolado de uma amostra de mel, porém o microrganismo-alvo foi o fungo *Macrophomina phaseolina*, espécie de importância apenas na fitopatologia. Kong et al. (2010) observaram que uma linhagem de *B. megaterium* de origem marinha foi capaz de inibir o crescimento de *A. flavus* e reduzir a produção de aflatoxinas. Neste caso, os autores não avaliaram outras espécies fúngicas produtoras de diferentes micotoxinas para confirmar o espectro de ação dessa bactéria.

Em relação à *Pseudomonas* sp. 4B, apesar da hipótese inicial sobre a capacidade desta bactéria em produzir uma bacteriocina, testes preliminares de precipitação do sobrenadante (obtido pelo cultivo celular) com sulfato de amônio, seguindo o protocolo descrito por Fontoura et al. (2009) não demonstraram resultados de inibição do crescimento fúngico. No entanto, uma vez que este gênero é reconhecido por produzir várias enzimas antifúngicas tais como quitinases, proteases e glucanases (CORDERO et al., 2012; AKOCAK et al., 2015; WU et al., 2016),

pesquisas sobre o potencial de *Pseudomonas* sp. 4B em produzir estas enzimas estão em andamento.

Análises de identificação molecular realizadas por Fontoura et al. (2009) indicaram que *Pseudomonas* sp. 4B apresenta 100 e 98% de similaridade com as espécies *Pseudomonas thermaerum* e *P. aeruginosa*, respectivamente. Pesquisas mais avançadas estão sendo finalizadas para confirmar a identificação dessa bactéria. Considerando essas duas espécies, os resultados referentes ao controle de fungos toxigênicos utilizando *Pseudomonas* sp. 4B podem ser vistos como promissores. Até o momento, não há relatos sobre quaisquer isolados de *P. thermaerum* com a capacidade de inibir o crescimento de fungos (independente do seu potencial toxigênico). Da mesma forma que, apesar de haver estudos demonstrando a atividade antifúngica de linhagens de *P. aeruginosa*, a influência dessa espécie sobre o crescimento de fungos produtores de micotoxinas nunca foi avaliada (WANG et al., 1999a, b; YEN et al., 2006; MARTIN-ARJOL et al., 2010; BORA, 2014; GORANTLA et al., 2014; GREWAL et al., 2014).

Outro aspecto importante durante o estudo destas bactérias como agentes de controle biológico está relacionado a sua influência sobre a síntese de micotoxinas. Uma hipótese que tem sido abordada na literatura é que as micotoxinas podem ser produzidas em resposta a um fator de stress, incluindo pela presença de outros microrganismos, o que leva a considerar que a redução do crescimento fúngico causada pela co-ocorrência de outros microrganismos poderia influenciar no aumento da síntese de micotoxinas (GAREIS; CEYNOWA, 1994). No entanto, o presente estudo demonstrou que *Bacillus* sp. P1, *Bacillus* sp. P11 e *Pseudomonas* sp. 4B não estimularam a produção de AFB1 e OTA. Tal fato poderia fornecer determinada vantagem destas bactérias para o controle de fungos toxigênicos. Ainda é importante avaliar o efeito destes isolados bacterianos sobre a síntese de outras micotoxinas para ampliar o potencial de ação destas bactérias, pois sabe-se que um mesmo isolado fúngico pode produzir mais de uma micotoxina (BRAGULAT et al., 2008; IAMANAKA et al., 2010; ROCHA et al., 2014).

A continuidade de pesquisas sobre o potencial antifúngico dessas bactérias pode contribuir para determinar suas possíveis aplicações. Microrganismos considerados agentes de controle biológico podem ser empregados em culturas agrícolas, onde bioformulações são aplicadas no rizosfera ou pulverizadas na

superfície de plantas e sementes. Desta forma, as bactérias protegem as culturas agrícolas através de interações antagonistas ou ainda por indução de resistência na planta, inibindo o crescimento de fungos toxigênicos que atacam antes da colheita (fungos de campo) (TROTEL-AZIZ et al., 2008; MANIKANDAN et al., 2010; DAMANN JR., 2015; ZHANG et al., 2016). Estudos ainda comentam que o uso de antagonistas microbianos durante a pós-colheita é mais promissor em comparação com sua utilização na pré-colheita devido às condições ambientais estáveis (TROTEL-AZIZ et al., 2008; DROBY et al., 2009; ZHANG et al., 2016). Neste caso, os produtos agrícolas são pulverizados ou imersos nas bioformulações, e com isso, ocorre a supressão do crescimento de fungos (CAVAGLIERI et al., 2005; JIANG et al., 2014; ZHANG et al., 2016) que se desenvolvem somente após a colheita, chamados de fungos de armazenamento (MILLER, 1995). Para confirmar a possibilidade de elaboração de novos produtos de controle biológico a partir dessas bactérias, a ação preventiva em produtos agrícolas de relevância, a avaliação da eficácia do controle biológico em casa de vegetação e em campo, além dos impactos ecológicos e toxicológicos de *Bacillus* spp. e *Pseudomonas* sp. 4B, estão entre os principais pontos a serem investigados em futuros trabalhos.

Em relação à segunda estratégia investigada neste estudo, o emprego de nanofibras de PCL funcionalizadas com cetoconazol e natamicina também apresentou resultados satisfatórios para o controle fúngico. A técnica de *electrospinning* permitiu a incorporação destes fármacos, conferindo às nanofibras capacidade antifúngica. Tal fato foi comprovado pela capacidade das membranas de nanofibras em formar halos de inibição de crescimento fúngico variando de 6 a 44 mm. A caracterização desses materiais também possibilitou verificar que a presença do cetoconazol ou natamicina não ocasionou mudanças expressivas na matriz polimérica das nanofibras.

Ainda foi possível observar que as nanofibras de PCL apresentaram a capacidade de liberação controlada dos antifúngicos incorporados. No caso das nanofibras funcionalizadas com cetoconazol, o perfil de liberação do fármaco nas soluções simulantes utilizadas foi contínuo durante o período de 72 horas de incubação e com valor máximo de acumulação nessas soluções de aproximadamente 87%. Da mesma forma, nanofibras de PCL incorporadas com natamicina também demonstraram liberação gradual do respectivo antifúngico. A funcionalidade dessas nanoestruturas foi confirmada quando alíquotas das soluções simulantes,

previamente mantidas em contato com as nanofibras, inibiram o crescimento dos fungos testados.

A possibilidade e eficácia de incorporação de vários compostos em nanofibras pelo processo de *electrospinning* têm despertado o interesse em vários ramos de pesquisa, incluindo na área de alimentos (CANBOLAT et al., 2014; REZAEI et al., 2015; RAJ; SHANKARAN, 2016). Além disso, algumas das principais características da poli- ϵ -caprolactona tais como, biodegradabilidade, hidrofobicidade e propriedades de liberação controlada de compostos funcionais justificam o interesse pela utilização desse polímero (WOODRUFF; HUTMACHER, 2010; RHIM et al., 2013). No entanto, ainda não há estudos na literatura sobre nanofibras de PCL incorporadas com cetoconazol e natamicina, sendo, portanto, o primeiro relato do desenvolvimento desses nanomateriais.

A administração oral do cetoconazol vem sendo descontinuada em vários países devido a sua toxicidade, impossibilitando seu uso em alimentos (YAN et al., 2013; GUPTA et al., 2015). Apesar disso, a adição deste antifúngico em nanofibras poderia permitir sua utilização como material de novos produtos farmacêuticos de uso tópico ou sistêmico para o tratamento clínico de infecções fúngicas. Levando em consideração a toxicidade do cetoconazol, como um dos problemas para a utilização em alimentos, é importante ressaltar que a elaboração dessas nanofibras também serve como modelo de estudo para novas pesquisas sobre a incorporação de outros compostos antifúngicos, especialmente aqueles que são permitidos em alimentos, como por exemplo a natamicina (EUROPEAN UNION, 1995; BRASIL, 1988, 2001; JECFA, 2002).

A utilização da natamicina como conservante alimentar é bastante conhecida devido ao amplo espectro de atividade contra fungos deteriorantes e toxigênicos (MEDINA et al., 2007b; EFSA, 2009; JUNEJA et al., 2012). Como citado anteriormente, sua hidrofobicidade pode dificultar a eficácia de aplicação nos alimentos (REPS et al., 2002; FUCINOS et al., 2012). Desta forma, é possível que a capacidade de liberação controlada da natamicina promova uma melhoria na distribuição desse composto, tornando sua ação mais efetiva. Por esse motivo, a adição de natamicina em nanoestruturas, como as nanofibras de PCL, contribui para o desenvolvimento de materiais com propriedades antifúngicas e potencial aplicação para embalagem de alimentos.

Embalagens com função antimicrobiana são altamente úteis para minimizar o crescimento de microrganismos contaminantes pós-processamento, estendendo a vida útil dos alimentos e mantendo a qualidade e segurança do produto. Exemplos específicos incluem embalagens antimicrobianas para o revestimento de produtos lácteos (principalmente queijo), produtos de panificação, cereais e frutas (RHIM et al., 2013; OTHMAN, 2014; VANDERROOST et al., 2014). Diante disso, ao considerar o interesse de aplicação das nanofibras funcionalizadas com natamicina como material antifúngico e antimicotoxigênico, uma avaliação sobre a utilização destas membranas na superfície de alimentos suscetíveis ao crescimento fúngico e à produção de micotoxinas se faz necessária. Ademais, análises atributos sensoriais (incluindo cor, sabor, textura, *off-flavor* e aceitabilidade geral) de alimentos embalados com esse material, bem como testes toxicológicos para confirmar a ausência de resíduos dos solventes utilizados na elaboração das nanofibras, são essenciais para a continuidade dessa pesquisa.

A produção de nanofibras de PCL funcionalizadas com cetoconazol e natamicina possibilitou demonstrar a viabilidade desse material para o controle do crescimento fúngico. Tais resultados também servem como referência para estimular futuras pesquisas sobre o desenvolvimento de nanofibras incorporadas com moléculas bioativas de origem microbiana, como por exemplo os metabólitos das bactérias aqui investigadas. Desta forma, a combinação de nanofibras de PCL com as substâncias antifúngicas produzidas por *Bacillus* spp. e *Pseudomonas* sp. 4B poderia criar novas perspectivas para os diferentes desafios referentes ao controle de fungos toxigênicos.

CONCLUSÃO

As estratégias avaliadas nesse estudo mostraram-se promissoras para controlar o desenvolvimento de fungos toxigênicos e a produção de micotoxinas. *Bacillus* spp. e *Pseudomonas* sp. 4B provenientes da Bacia Amazônica e de um lago de tratamento de efluentes, respectivamente, apresentaram atividades antagonistas contra fungos toxigênicos, influenciando nos parâmetros de crescimento fúngico que foram observados através da redução na taxa de crescimento micelial, esporulação e germinação de esporos, bem como na diminuição da produção de micotoxinas (AFB1 e OTA). Além disso, algumas linhagens do gênero *Bacillus* foram capazes de produzir lipopeptídeos, incluindo iturina A e surfactinas, que podem ter contribuído para a ação antifúngica dessas bactérias.

As nanofibras de PCL funcionalizadas com cetoconazol e natamicina também podem contribuir para o controle de fungos produtores de micotoxinas. O desenvolvimento de nanofibras pela técnica de *electrospinning* permitiu a incorporação dos respectivos fármacos sem alterar as principais características morfológicas (diâmetro das fibras) e químicas (espectros de absorção de grupos químico específicos de PCL) das nanoestruturas, além de manter a atividade antifúngica dos compostos. Desta forma, o processo de *electrospinning* possibilitou a incorporação de determinada função antimicrobiana às nanofibras de PCL devido às propriedades de liberação controlada dos antifúngicos.

Diante disso, é possível sugerir que as bactérias e o nanomateriais investigados neste estudo são ferramentas promissoras para o desenvolvimento de estratégias de controle de fungos toxigênicos e produção de micotoxinas. Pesquisas sobre a prevenção ou redução da síntese de micotoxinas em diferentes matérias-primas e produtos industrializados suscetíveis à contaminação fúngica devem ser realizadas para avaliar a aplicabilidade dessas opções antimicotoxigênicas.

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