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The *Penicillium digitatum* antifungal protein PdAfpB shows high activity against mycobiota involved in sliced bread spoilage

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ABSTRACT

This study aimed to isolate and identify fungal species involved in sliced bread spoilage, and to evaluate their susceptibility to antifungal proteins of fungal origin (AFPs). Proteins include PdAfpB from *Penicillium digitatum* and PeAfpA, PeAfpB and PeAfpC from *Penicillium expansum*. Based on morphological criteria, a group of sixteen fungal isolates were selected and subsequently identified at the species level using sequence analysis. *Penicillium* species, the predominant mycobiota, were identified based on a combined phylogenetic analysis using ITS and β-tubulin sequences, being *P. roqueforti*, *P. brevicompactum*, *P. chrysogenum* and *P. crustosum* the most abundant species. *Aspergillus versicolor*, *Aspergillus niger* and *Bissochlamys spectabilis* were also identified. Regarding the antifungal activity of AFPs, PdAfpB and PeAfpA were the most potent proteins since the growth of most of tested fungi was completely inhibited by concentrations ranging from 2 to 32 μg/mL. PeAfpB showed moderate antifungal activity, whereas PeAfpC was the least active protein. The best *in vitro* AFPs, PdAfpB and PeAfpA, were also evaluated in *in situ* protection assays against *P. roqueforti*. PdAfpB provoked a clear reduction of *P. roqueforti* growth in sliced bread samples, suggesting that this AFP has a protective effect on bread. This study underlines the potential of the AFPs tested, in particular PdAfpB, as alternative antifungal agents for extending sliced bread shelf life.

1. Introduction

Sliced bread is one of the most susceptible bakery products for fungi spoilage, resulting in significant economic losses for the bakery industry but also food waste at the consumer level (Magan et al., 2003; Melikoglu and Webb, 2013). The percentage of spoiled products can reach up to 50% (Nguyen Van Long et al., 2016) and it is mainly caused by *Penicillium* spp., followed by *Aspergillus*, *Cladosporium*, *Mucor* and *Fusarium* genera (Dagnas et al., 2014; Ju et al., 2020a, 2020b). Apart from the unpleasant sight of visible fungal growth and off-flavors, some fungi are also responsible for the production of mycotoxins, which represent a hazard to the health of consumers (Liu et al., 2020).

Several methodologies, aiming to reduce fungal contamination and to extend the shelf life of bakery products are currently being applied. They include modified atmosphere packaging, irradiation, aseptic packaging and the addition of salts of organic acids such as sorbate, benzoate, and propionate, in particular calcium propionate (Axel et al., 2017; Belz et al., 2012; Garcia and Copetti, 2019). Among them, chemical preservatives show some advantages such as a wide spectrum activity, low toxicity and relatively low cost. Although they are classified as generally recognized as safe (GRAS) compounds (Schmidt-Heydt et al., 2007), some studies have shown that they are linked to diverse adverse effects, including attention deficit hyperactivity disorder and allergic reactions (Dey and Nagababu, 2022; Sasaki et al., 2002). In Europe, maximum limits for these preservatives have been established in the range of 1000–3000 mg/kg (European Union, 95/2/EC, 1995), which, sometimes, is not sufficient for the prevention of bread rot (Suhr and Nielsen, 2004). Moreover, the use of sublethal doses of these chemical preservatives may lead to stimulation of mycotoxin biosynthesis by fungi (Alcano et al., 2016; Arroyo et al., 2005; Marín et al., 2002).

Current research aims to extend the shelf life of bread through the

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use of more consumer-friendly preservatives as alternatives to chemical additives (Axel et al., 2017; Ju et al., 2020a). In this context, antifungal proteins (AFPs) secreted by filamentous fungi could represent new biopreservatives (Thery et al., 2019). AFPs are small, cationic, cysteine-rich proteins that fold into compact structures, which makes them highly stable to pH, temperature, and proteolysis (Batta et al., 2009). AFPs exhibit broad antifungal spectra and different mechanisms of action against filamentous fungi, which makes the development of fungal resistance unlikely (Delgado et al., 2019a; Garrigues et al., 2016; Hegedüs and Marx, 2013; Marx et al., 2008). Moreover, additional functions of AFPs are associated to different biological processes including the reduction of mycotoxins biosynthesis (Martínez-Culebras et al., 2021a). These features, and in particular the thermostability, have attracted the interest of the food industry in AFPs as new food preservatives (Delgado et al., 2016; Leyva Salas et al., 2017; Shwaiki et al., 2021).

In previous studies, AFPs from *P. digitatum* (PdAfpB) and *P. expansum* (PeAfpA, PeAfpB and PeAfpC), were characterized and their distinctive antifungal profiles were demonstrated (Garrigues et al., 2017, 2018). These AFPs show significant activity against a representative panel of mycotoxin-producing fungi belonging to the genera *Alternaria*, *Aspergillus*, *Byssochlamys*, *Fusarium* and *Penicillium* (Martínez-Culebras et al., 2021b). This study aims to isolate and identify fungal isolates from sliced bread samples, and to carry out *in vitro* and *in situ* experiments to evaluate the antifungal activity of these active AFPs against the contaminant mycobiota.

2. Material and methods

2.1. Samples and fungal isolation

Twelve commercial samples of different kind of sliced bread were randomly purchased from different supermarkets in the area of Burjassot and Paterna (Valencia, Spain) with valid expiration date of consumption. After a period of 30 days of storage at 25 °C, fungal strains were isolated from spoiled loaf samples and plated on Dichloran rose bengal chloranphenicol (DRBC) medium (Scharlau Chemie, S.A., Spain) (Pitt and Hocking, 2009). Fungal colonies exhibiting distinct morphotypes were isolated and transferred to Malt Extract Agar (Scharlau Chemie, S. A.) (MEA) plates to obtain pure cultures.

2.2. Phenotypic identification

Twenty-three fungal isolates were named as PM1-PM23, respectively and identified through macroscopic and microscopic observation, with the aid of published guidelines (Frisvad and Samson, 2004; Pitt and Hocking, 2009; Samson et al., 2004). For macro-morphological observations, isolates were three-point inoculated on 4 standardized solid culture media: Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Yeast Extract Sucrose Agar (YES) and Czapek Yeast Extract Agar (CYA) (Schlau Chemie, S.A.) (Samson et al., 2004). Cultures were incubated at 25 °C for 7 days in the dark. For the determination of micro-morphological characteristics, microscope slides were prepared and examined. Fungal isolates from the same sample that also showed similar macroscopic and microscopic characteristics were considered the same group. Selected fungal isolates from each one of these similar fungal groups were additionally identified at the species level by sequence analysis.

2.3. ITS and β-tubulin gene amplification and sequence analysis

Fungal DNA extraction was performed as described by Khang et al. (2006). ITS and β-tubulin gene *BenA* from selected sliced bread isolates (see Table 1) and reference strains of *Penicillium* were obtained using the its5/its4 (White et al., 1990) and Bt2a/Bt2b (Carbone and Kohn, 1999) primers, respectively. The ITS and β-tubulin gene sequences of *Penicillium aurantiogriseum* CBS 324.89 (JN942751; MN969372), *Penicillium brevicompactum* NRRL 2012 (AY484913; DQ645785), *Penicillium carneum* CBS 112297 (MH86289; AY674386), *Penicillium chrysogenum* DTO 10294 (JX996998; JF909959), *Penicillium citrinum* DTO 390–16 (MN788117; MN787907), *Penicillium commune* QP3 (MK660354; MK675758), *Penicillium crustosum* DI16-101 (LT558923; LT559041), *Penicillium expansum* YC-IK11 (MK850332; MK862430), *Penicillium griseofulvum* CBS 185.27 (MH866419; JF909942), *Penicillium italicum* ATHUM 3004 (FJ004301; FJ004417), *Penicillium olsoni* NRRL 35612 (DQ645803; DQ645801), *Penicillium paneum* CBS 101032 (MH862717; AY674387), *Penicillium polonicum* DI16-104 (LT558926; LT559044), *Penicillium roqueforti* DI16-81 (LT558903; LT55902) and *Penicillium rubens* DI16-44 (LT558866; LT55894) were obtained from the EMBL (European Molecular Biology Laboratory) sequence database. PCR reactions were performed in a total volume of 25 μL containing 50 ng of DNA, 10 mM Tris-HCl, 80 μM (each) dNTPs, 1 μM of each primer, 2 mM of $MgCl_2$ and 1 U of DNA polymerase (BIOTAQTM DNA polymerase). The reaction mixtures were incubated for 35 cycles of 30 s at 94 °C, 1 min at 52 °C and 1 min at 72 °C in a thermocycler (Labnet, Multigene, Merck, Spain). The its5/its4 and Bt2a/Bt2b primer pairs were also used to obtain the sequence of both strands of ITS and *BenA* sequences, respectively.

PCR products were directly sequenced by using the sequencing kit Taq DyeDeoxy (Applied Biosystems, Falmer, Brighton, United

Table 1
Minimal inhibitory concentration (MIC) (μg/mL) and minimal fungicidal concentration (MFC) values of AFPs against sliced bread spoilage isolates.

Fungi	Code	Genbank Accession Number		PeAfpA			PeAfpB			PeAfpC			PdAfpB		
		ITS	β-tubulin	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R
A. niger	PM6	ON175935	ND	2	2	1	32	32	1	>	>		4	4	1
A. versicolor	PM7	ON175936	ND	>	>		>	>		>	>		16	16	1
B. spectabilis	PM4	ON175937	ND	2	4	2	>	>		4	8	2	2	8	4
P. brevicompactum	PM1	ON116657	ON155591	4	4	1	>	>		>	>		4	4	1
P. brevicompactum	PM15	ON116664	ON155598	8	8	1	>	>		>	>		8	8	1
P. brevicompactum	PM20	ON116667	ON155601	8	8	1	>	>		>	>		8	16	2
P. chrysogenum	PM5	ON116659	ON155593	4	4	1	>	>		>	>		4	4	1
P. chrysogenum	PM12	ON116662	ON155596	4	4	1	8	8	1	>	>		4	4	1
P. chrysogenum	PM16	ON116665	ON155599	32	32	1	>	>		>	>		16	16	1
P. commune	PM22	ON116668	ON155602	4	16	4	>	>		>	>		2	8	2
P. crustosum	PM3	ON116658	ON155592	4	4	1	32	32	1	>	>		4	4	1
P. crustosum	PM8	ON116660	ON155594	2	8	4	16	16	1	>	>		2	4	2
P. crustosum	PM23	ON116669	ON155603	2	4	2	>	>		>	>		4	4	1
P. expansum	PM14	ON116663	ON155597	4	8	2	32	32	1	>	>		4	4	1
P. roqueforti	PM9	ON116661	ON155595	2	4	2	>	>		>	>		2	2	1
P. rubens	PM18	ON116666	ON155600	4	4	1	>	>		>	>		4	8	2

ND sequence not determined, "> " represents a non-inhibited growth at the maximum concentration of AFPs used (32 μg/mL) and "R" the MFC/MIC ratio.

Kingdom). PCR reactions were performed in a final volume of 10 μL, containing 20–30 ng of DNA, 5 × reaction buffer (BigDye Terminator), 3,2 μM of each primer and 1U of the enzyme PREMISC. Sequencing PCR conditions involved 99 cycles of 10 s at 96 °C, 10 s at 52 °C and 4 min at 60 °C. PCR products were sequenced on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, USA) in the Central Service for Experimental Research (SCSIE) of the University of Valencia. Chromatograms were analyzed using the MEGA X version 4.1 software (Kumar et al., 2018).

Sequences were aligned using the program MEGA X version 4.1 (Kumar et al., 2018). The genetic distances were calculated using the Jukes–Cantor model and the phylogenetic inference was obtained by the neighbour-joining (NJ) method (Saitou and Nei, 1987). The NJ tree and the statistical confidence of a particular group of sequences in the tree, evaluated by bootstrap test (1000 pseudoreplicates) (Hillis and Bull, 1993), were also performed using the MEGA X version 4.1 software (Kumar et al., 2018).

2.4. Antifungal activity assays

Growth inhibition assays were performed in 96-well, flat-bottom microtiter plates (Nunc, Roskilde, Denmark) based on the broth microdilution method as previously described (Garrigues et al., 2017), with minor modifications. Briefly, 50 μL of fungal conidia (5 x 104 conidia/mL) in 10% PDB (PDB; Scharlau Chemie, S.A., Spain) containing 0.02% (w/v) cloramphenicol (Sigma, Spain) to avoid bacteria contamination were mixed with 50 μL of twofold concentrated proteins from serial twofold dilutions (final concentrations ranging from 0 to 32 μg/ml. PdAfpB from *P. digitatum* and the three PeAFPs from *P. expansum* were produced and purified as previously described (Garrigues et al., 2018; Hernanz-Koers et al., 2018). In order to avoid evaporation, plates were incubated inside a box on top of water-saturated filter paper. Plates were statically incubated for 72 h at 25 °C. Growth was determined every 24 h by measuring the optical density (OD) at 600 nm using a FLUOstar Omega plate spectrophotometer (BMG labtech, Orlenberg, Germany), and the OD_{600} mean and standard deviation (SD) of three replicates were calculated. Dose-response curves were generated from measurements after 72 h. These experiments were repeated at least twice for each protein-isolate combination. Minimum inhibitory concentration (MIC) is defined as the lowest tested concentration of AFPs that prevented detectable growth (OD measurements below the signal of the blank non-inoculated controls) at the end of the incubation period (72 h) in all the experiments conducted. Minimal fungicidal concentrations (MFC) were determined by spotting 10 μL of each well of 96-well plates showing absence of growth on 24-well PDA plates. After incubation at 25 °C for 72 h, the lowest dilution with no growth indicated the MFC. To assess the fungicidal and fungistatic activity, the MFC/MIC ratio was used. A ratio of ≤ 4 was considered fungicidal, whereas a ratio >4 was considered fungistatic (Hazen, 1998; Pfaller et al., 2004).

2.5. In situ antifungal analysis on sliced bread samples

Two replicates of commercial sliced bread without additives (white wheat natural Bimbo, Bimbo Donuts Iberia S.A.U. Madrid, Spain), were inoculated with 50 μL of *P. roqueforti* conidial suspension (1×10^4 conidia/mL) and 100 μg/mL of PeAfpA or PdAfpB. Bread slices were cut out and placed into sterile Petri dishes. Each bread slice was point inoculated with either fresh conidia suspension of *P. roqueforti* mixed with each protein (in duplicate), or conidia without the protein (control). Bread samples were incubated at 25 °C. The antifungal effect was evaluated by visual inspection for growth development on consecutive days post inoculation (dpi). PdAfpB at 25 μg/mL and 50 μg/mL was additionally tested against *P. roqueforti* following this procedure.

3. Results

3.1. Fungal isolation from sliced bread and species identification

A total of 23 fungal isolates from sliced bread (PM1-PM23) were firstly identified at the genera level using traditional criteria (see Material and methods). Predominant mycobiota belonged mainly to the genera *Penicillium* (20 isolates, 81.95% of total isolates) followed by *Aspergillus* (2 isolates, 8.69%) and *Byssochlamys* (1 isolate, 4.35%). Based on morphological and cultural comparisons, the 23 isolates were categorized in 16 groups. One representative of each group was selected for molecular identification at the species level using sequencing analysis. Results confirmed that most of the sliced bread-isolated fungi identified were *Penicillium* spp. (13 out of 16 fungal isolates). *Penicillium* spp isolates were identified to the species level based on a combined phylogenetic analysis using ITS and β-tubulin sequences (Fig. 1; Table 1). Phylogenetic analysis grouped the *Penicillium* spp isolates in two well separated clusters (Fig. 1). Most of the isolates (10 out of 13) were contained within a well-supported cluster I (bootstrap value, 95). Within this cluster I, isolates PM3, PM8 and PM23 were identified as *P. crustosum* while PM5, PM12 and PM16 were identified as *P. chrysogenum*. The remaining isolates were identified as *P. commune* (PM22), *P. rubens* (PM18), *P. expansum* (PM14) and *P. roqueforti* (PM9). On the other hand, isolates PM1, PM15 and PM20 were identified as *P. brevicompactum* within a well-supported cluster II (bootstrap value, 99). Overall, *P. roqueforti* and *P. chrysogenum* were the most predominant fungal species with 4 isolates each (17, 3%), followed by *P. brevicompactum* and *P. crustosum* (3 isolates each, 13%).

Additionally, fungal isolates different to *Penicillium* spp. were identified using ITS sequencing and BLAST on the NCBI website (www.ncbi.nlm.nih.gov/BLAST/). One isolate from each one of the species *Aspergillus versicolor* (PM7), *Aspergillus niger* (PM6) and *Byssochlamys spectabilis* (PM4) were identified (Table 1).

3.2. Antifungal activity profiles of AFPs

The three PeAFPs and PdAfpB were tested for their antifungal activity against the 16 identified fungal isolates (Table 1). Differences in antifungal activity were observed among the four AFPs tested (Table 1 and Fig. 2). Table 1 shows MIC and MFC values of each AFP against all fungal species evaluated, whereas Fig. 2 shows representative dose-response curves comparing the antifungal activity of the four AFPs. Among the proteins evaluated, the most active AFPs were PdAfpB and PeAfpA with similar activity, followed by PeAfpB and PeAfpC, respectively.

PdAfpB was able to inhibit the growth of all tested fungi. MIC values varied from 2 μg/mL against *B. spectabilis* PM4, *P. commune* PM22, *P. crustosum* PM8 and *P. roqueforti* PM9 to 16 μg/mL against *A. versicolor* PM7 and *P. chrysogenum* PM16. Most of the PdAfpB MIC values were in the range of 2–4 μg/mL (Table 1). PeAfpA also exhibited a broad spectrum of activity, and it was able to inhibit the growth of all tested fungi with the exception of *A. versicolor* PM7, which was not totally inhibited at the highest concentration tested (32 μg/mL). MIC values ranged from 2 μg/mL against *A. niger* PM6, *B. spectabilis* PM4, *P. crustosum* PM8 and PM23 and *P. roqueforti* PM9 isolates to 32 μg/mL against *P. chrysogenum* PM16. PeAfpB showed limited antifungal activity and inhibited only five of the tested fungi, with MIC values ranging from 8 μg/mL against *P. chrysogenum* PM12 to 32 μg/mL against *P. crustosum* PM3 and *P. expansum* PM14. MIC values were not reached, in the conditions tested, against *A. versicolor* PM7, *B. spectabilis* PM4, the three *P. brevicompactum* isolates PM1, PM15 and PM20, two out of the three *P. chrysogenum* isolates (PM5 and PM16), *P. commune* PM22, *P. crustosum* PM23, *P. roqueforti* PM9 and *P. rubens* PM18. Finally, PeAfpC was the least active protein, only inhibiting the growth of *B. spectabilis* PM4 (MIC value of 4 μg/mL).

Most susceptible isolates to the action of AFPs were *B. spectabilis*

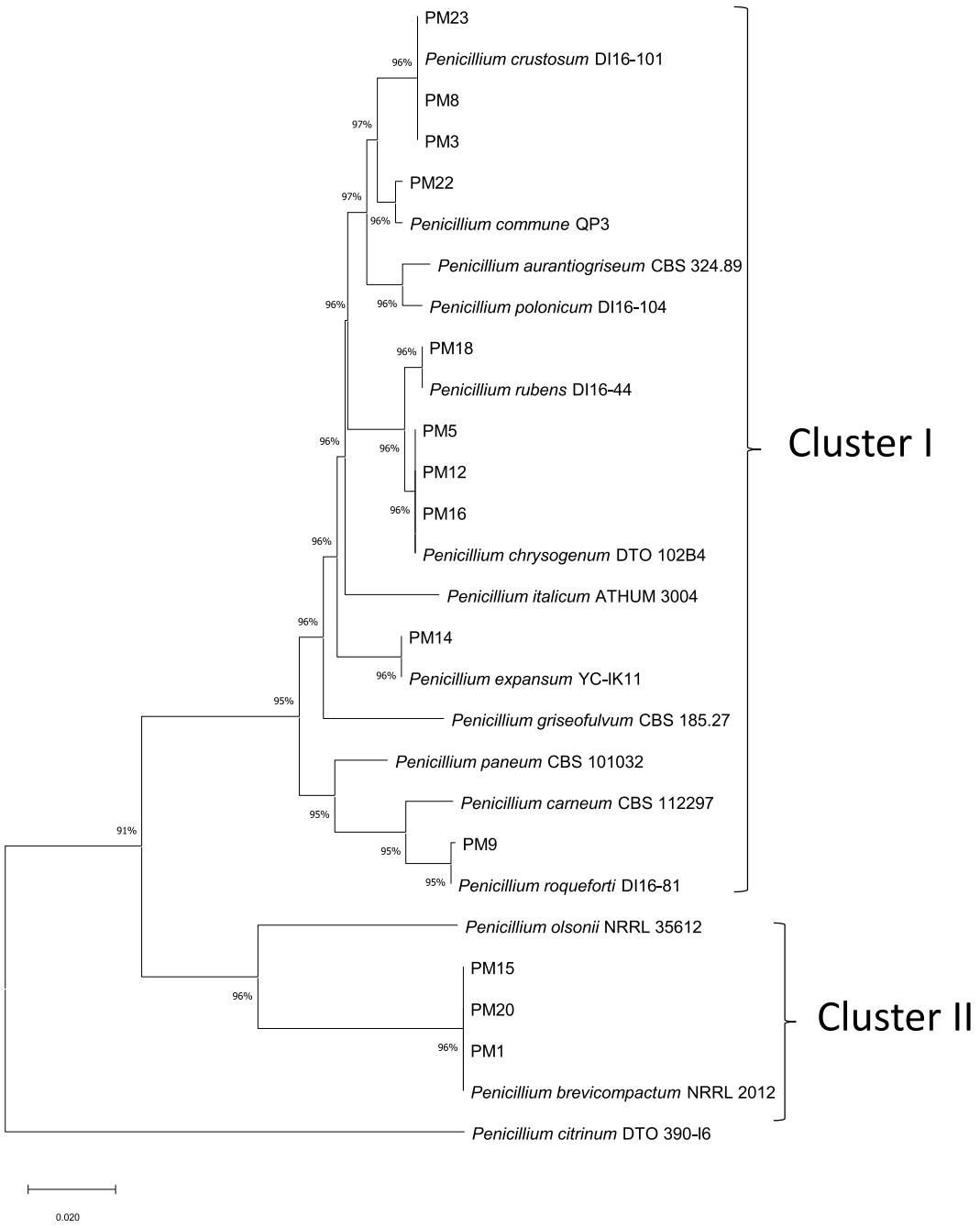

Fig. 1. Neighbour-joining tree based on nucleotide divergences, estimated according to Jukes–Cantor model, from the combined datasets of ITS and β-tubulin sequences. The numbers on the nodes are the frequency (in percent) with which a cluster appears in a bootstrap test of 1000 runs.

PM4, *P. crustosum* PM8 and *P. roqueforti* PM9, for which MIC values of proteins PeAfpA and PdAfpB were 2 μg/mL. In contrast, isolates more tolerant to AFPs were *A. versicolor* PM7 and *P. chrysogenum* PM16 as shown in Table 1.

Fungal isolates for which MIC values were reached in liquid medium were also subjected to MFC determination (Table 1). MFC values of PeAfpA and PdAfpB were slightly higher than their respective MIC values and ranged between 2 and 32 μg/mL. Regarding PeAfpB, MFC and MIC values were identical (in the range 8–32 μg/mL). Finally, MFC of PeAfpC for *B. spectabilis*, the only fungus that was totally inhibited by this protein, was 8 μg/mL. Since no relevant differences between MIC and MFC values were observed, the MFC/MIC ratio for most of the fungi tested ranged between 1 and 2. By extrapolation from the conventional definition used for yeast (Hazen, 1998; Pfaller et al., 2004), the four AFPs acted with fungicidal mechanisms towards all fungal isolates tested, since all the MFC/MIC ratios were below 4.

3.3. *In situ antifungal assays on sliced bread samples*

Based on the *in vitro* antifungal susceptibility tests, experiments were designed to evaluate the ability of the two most active proteins, PdAfpB and PeAfpA, to control the growth of *P. roqueforti*, the major responsible of bread spoilage (Garcia et al., 2019c), and one of the most prevalent fungal species found in the present study. A first experiment was set up to evaluate the effect of AFPs at a concentration of 100 μg/mL on *P. roqueforti* growth in commercial sliced bread (Fig. 3A). Effectiveness of proteins was registered based on visible fungal growth development. Along the experiment, PeAfpA showed a very slight growth reduction

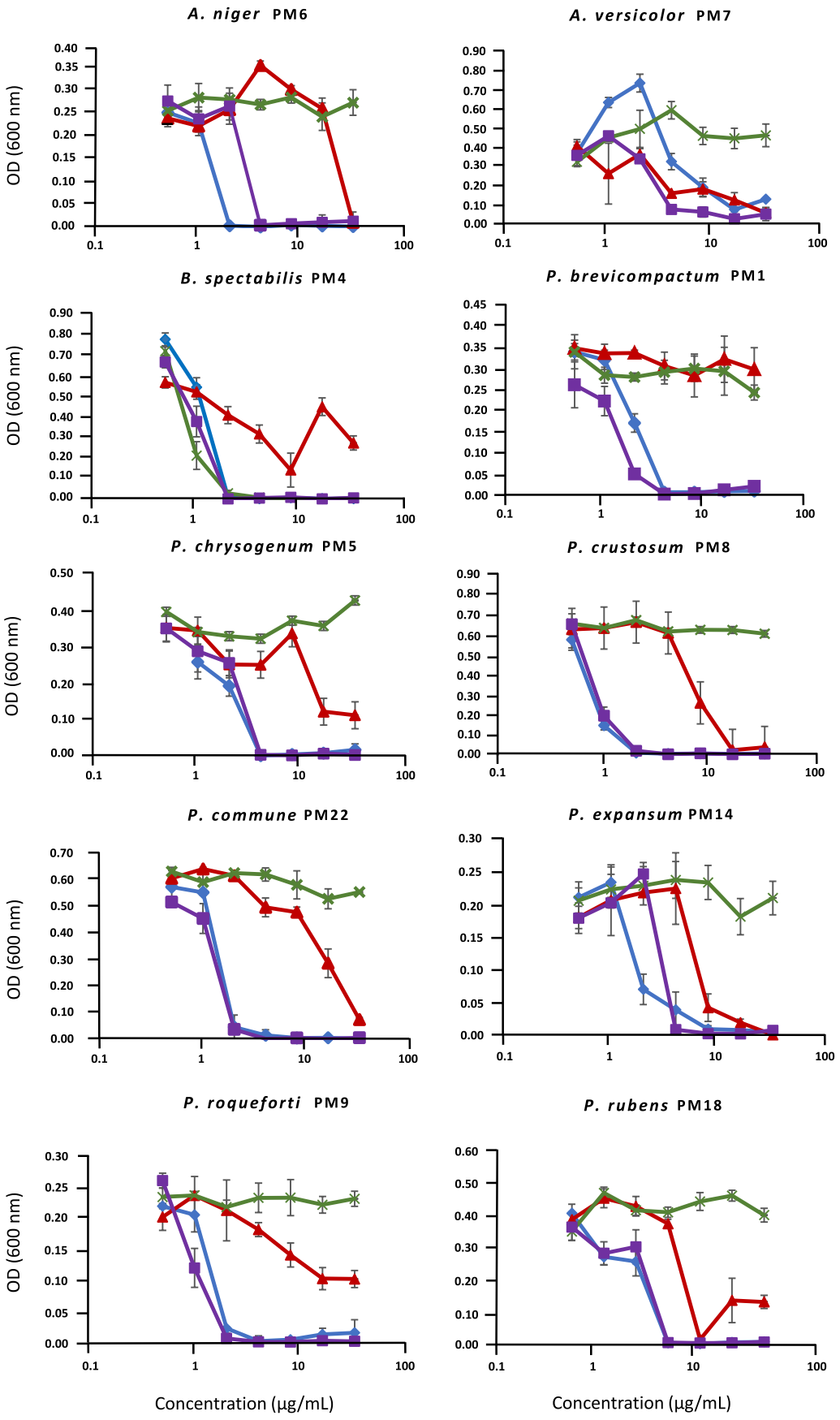

Fig. 2. Dose-response curves comparing the antifungal activity of PeAfpA (blue diamonds), PeAfpB (red triangles), PeAfpC (green cross) and PdAfpB (purple squares) against bread spoilage fungi. Dose-response curves show mean OD_{600} ± SD of triplicate samples after 72 h at 25 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

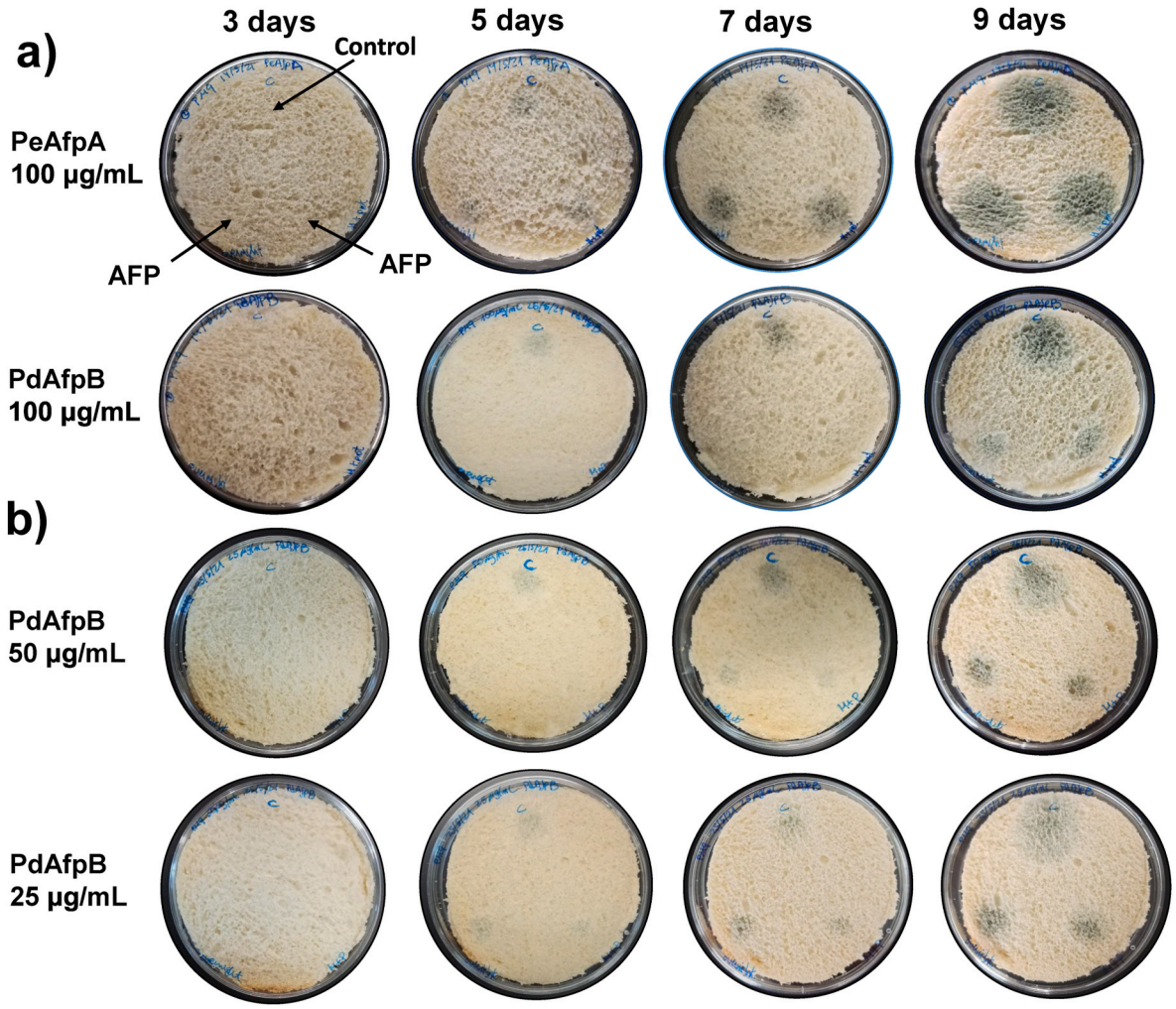

Fig. 3. Effect of AFPs on the fungal contamination of sliced bread artificially inoculated with a conidial suspension (1×10^4 conidia/mL) of *P. roqueforti*. Each bread slice was three-point inoculated with a control (conidia without protein), and two fresh conidia suspension mixed with each protein. (a) Effect of PeAfpA and PdAfpB at 100 µg/mL on the fungal growth development on sliced bread. (b) Effect of PdAfpB at 50 and 25 µg/mL on the fungal growth development on sliced bread.

compared to the control with no AFP treatment. However, PdAfpB treatments resulted in a lack of fungal growth at 5 and 7 dpi, and a clear delay of fungal growth at 9 dpi in comparison to the control. With the aim of confirming the efficacy of PdAfpB in controlling sliced bread spoilage by *P. roqueforti*, a second inoculation experiment was designed using lower concentrations of PdAfpB (50 and 25 µg/mL) (Fig. 3B). Although the concentration of 100 µg/mL was more efficient in controlling fungal growth, PdAfpB at 50 and 25 µg/mL was also effective in reducing the growth of *P. roqueforti* PM9 compared to the control. Fungal growth was not visible at 5 dpi using 50 µg/mL, whereas some growth was observed using 25 µg/mL. At 7 dpi, fungal growth development was reduced at both concentrations.

4. Discussion

Fungal spoilage is the main cause of substantial economic losses in packaged bakery products and food waste at the consumer level, and the use of preservatives is required for obtaining the necessary shelf life under ambient storage conditions (Axel et al., 2017; Nguyen Van Long et al., 2016). Here, based on morphological criteria, a selected group of 16 fungal isolates from commercial sliced bread samples, were identified at the species level using sequence analysis. Among them, *P. roqueforti, P. chrysogenum*, *P. brevicompactum,* and *P. crustosum* were the most prevalent species, which is in accordance with literature (Garcia et al., 2019a; Garcia et al., 2019b; Northolt, M.D. et al., 1995; Pitt and Hocking, 2009; Santos et al., 2016). Their occurrence in raw materials may contribute to further contamination of the processing environment, since these fungi produce a high amount of spores (Santos et al., 2016). *P. commune* has also been associated with bread spoilage (Santos et al., 2016; Suhr and Nielsen, 2004). By contrast, *P. expansum* and *P. rubens* are not commonly isolated from bread. *P. expansum* is mainly associated with pome fruit spoilage, while *P. rubens* is a common fungus of indoor environments (Houbraken et al., 2011; Luciano-Rosario et al., 2020). Our data suggest that both *Penicillium* species could form part of the common mycobiota that contaminates sliced bread. On the other hand, *P. polonicum* and, in particular, *P. paneum*, which are also principal fungal species from spoiled bread (Garcia et al., 2019c; Santos et al., 2016), were not isolated in this study. *A. niger* and the heat resistant *B. spectabilis* identified in this study are amongst the most common species involved in the spoilage of bread (Debonne et al., 2020; Garcia et al., 2021; Santos et al., 2016). Also, some mycotoxin-producing fungi were isolated in the present study. For instance, *A. versicolor* produces sterigmatocystin and aflatoxins, *A. niger* synthesizes ochratoxin A, and *P. expansum* and *B. spectabilis* generates patulin. Other minor mycotoxins are produced by *P. commune* (cyclopiazonic acid) and by *P. roqueforti* and *P. crustosum* (roquefortine C) (Perrone and Susca, 2017). Further studies are needed to determine the involvement of the major fungal spoilage species in mycotoxin production.

AFPs were effective at the concentrations tested (1–32 µg/mL) against the 16 fungal isolates associated with bread spoilage, although

considerable differences in effectiveness could be observed among the proteins. These AFPs differ in amino acid composition, primary structure and physicochemical properties (Garrigues et al., 2017, 2018), which might explain these different antifungal profiles observed. Here, the antifungal efficiency of PdAfpB against slice bread spoilage fungi is very similar to that of PeAfpA. These results are not totally in accordance with our previous studies, where PeAfpA was more effective than PdAfpB against a set of different *Penicillium*, *Aspergillus* and *Byssochlamys* species, suggesting a strain-dependency effect of AFPs. Finally, the activity of PeAfpC towards *B. spectabilis* is in agreement with our previous study (Martínez-Culebras et al., 2021b). It is interesting to note that *B. spectabilis* can pose a risk to product shelf life due to their heat resistance and high tolerance to low oxygen levels and to preservatives such as propionic acid and/or sorbates (Pitt and Hocking, 2009). Further studies are needed to assess the antifungal activity of AFPs against a larger number of *B. spectabilis* isolates from sliced bread.

Differences in susceptibility to AFPs were observed among fungal species (Table 1), suggesting that the resistance behavior to AFPs was species-dependent. This is in agreement with our previous study (Martínez-Culebras et al., 2021b), in which differences in susceptibility among mycotoxin-producing species were also observed. Likewise, the antifungal effect against mycotoxigenic fungi of PgAFP from *P. chrysogenum* (Rodríguez-Martín et al., 2010), differed not only between species of the same genera, but also within the same species (Delgado et al., 2015, 2019b). Here, some differences among isolates from the species *P. brevicompactum*, *P. chrysogenum* and *P. crustosum* were observed. Further studies of susceptibility and resistance of fungal species including more strains from each species will be needed to elucidate the antifungal specificities of AFPs.

Our results suggest that the tested AFPs might exhibit a fungicidal effect. These are promising results considering that chemical preservatives used in the bakery industry are fungistatic compounds, and consequently high concentrations are needed to control fungal growth. The use of high doses of synthetic preservatives may adversely affect the organoleptic properties of the bread, and the prolonged and repetitive treatment accelerates the development of resistance against these additives, as found for *P. roqueforti* (Garcia et al., 2021; Suhr and Nielsen, 2004). Moreover, the use of preservatives at a concentration lower than the recommended may facilitate both fungal growth and mycotoxin production. For instance, *P. verrucosum* growth and OTA production was stimulated by decimal reductions of preservatives (Arroyo et al., 2005; Schmidt-Heydt et al., 2007). Accordingly, the use of AFPs with a fungicide effect could be a promising solution for controlling fungal spoilage and prevent mycotoxin contamination. However, more comprehensive studies are needed to evaluate the fungicidal or fungistatic effect of AFPs *in situ*. Further research about the effect of AFPs on mycotoxin production are needed.

Here, we have shown that PdAfpB exerted protective effect against *P. roqueforti* in sliced bread. By contrast, PeAfpA identified as an *in vitro* highly active protein against *P. roqueforti* (MIC = 2; Table 1), did not showed any effect in bread. Discrepancies between *in vitro* and *in vivo* antifungal efficacies in oranges and apple fruits using these and other AFPs have been described by Gandía et al. (2021). In *in situ* experiments, additional factors that are absent in or differ from *in vitro* assays impact the antifungal potential of AFPs. For instance, specific substrates from raw material may influence fungal growth and AFP susceptibility. Compounds added in the process of breadmaking may also interfere with the AFP activity. Moreover, effectiveness in the reduction of the growth of *P. roqueforti* was observed in treatments with 50 and 25 μg/mL of PdAfpB. This indicates that small amounts of AFPs could be successfully used to control fungal spoilage of sliced bread. Our study emphasizes the need for assays in food matrices, as those described here, to evaluate the feasibility of AFPs in the control of bread spoilage fungi.

The interest in applying antifungal proteins and peptides for bread biopreservation has increased in recent years (Axel et al., 2017; Coda et al., 2008; Gobbetti et al., 2020; Rizzello et al., 2015, 2017). Antifungal peptide-producing lactic acid bacteria (LAB) have been used to extend the shelf life of bakery products (Axel et al., 2014; Nasrollahzadeh et al., 2022; Ryan et al., 2008, 2009). Protein legume extracts have also been successfully used for prolonging the shelf life of baked goods (Rizzello et al., 2011, 2015, 2017). (Rizzello et al., 2017). More recently, hydrolysates of goat milk whey and wheat gluten have also been used to extend the shelf life of bread (Freitas et al., 2022; Luz et al., 2020a, 2020b). To our knowledge, there are no studies on the evaluation of purified fungal AFPs as preservatives to extend the shelf life of bakery products.

Considering the potent and the broad *in vitro* inhibition spectrum of the AFPs tested, and the strong inhibition *in situ* of *P. roqueforti* by PdAfpB, it seems likely that this protein might also be effective in extending the shelf life of bakery products. Nevertheless, the antifungal activity evaluation of PdAfpB against other fungi is limited and more fungal species need to be investigated *in situ*. The efficient biotechnological production of PdAfpB achieved by Garrigues et al. (2017) could guarantee the production of stable, pure, and active protein in quantities required for successful application. PdAfpB shows notable ability to withstand protease and thermal degradation while it does no show hemolytic activity (Garrigues et al., 2017), suggesting it can be used as a bakery ingredient. Thus, PdAfpB is a promising alternative to the chemical preservatives currently used for the control of bread spoilage. Moreover, effectiveness of PdAfpB may also benefit from effective synergistic combinations with other fungicides agents or other control strategies (Melini and Melini, 2018). Additionally, delivery systems such as incorporation in biofilms may avoid proteolytic degradation or interaction with foods ingredients as described for the cationic peptide E-Poly-L-lysine, which incorporation in biofilms has shown to be successful to extend the shelf life of bread (Luz et al., 2018). Future efforts will be directed to study different PdAfpB application methods to extend the shelf-life of sliced bread products.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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