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Original Article

Antifungal proteins against cheese spoilage fungi

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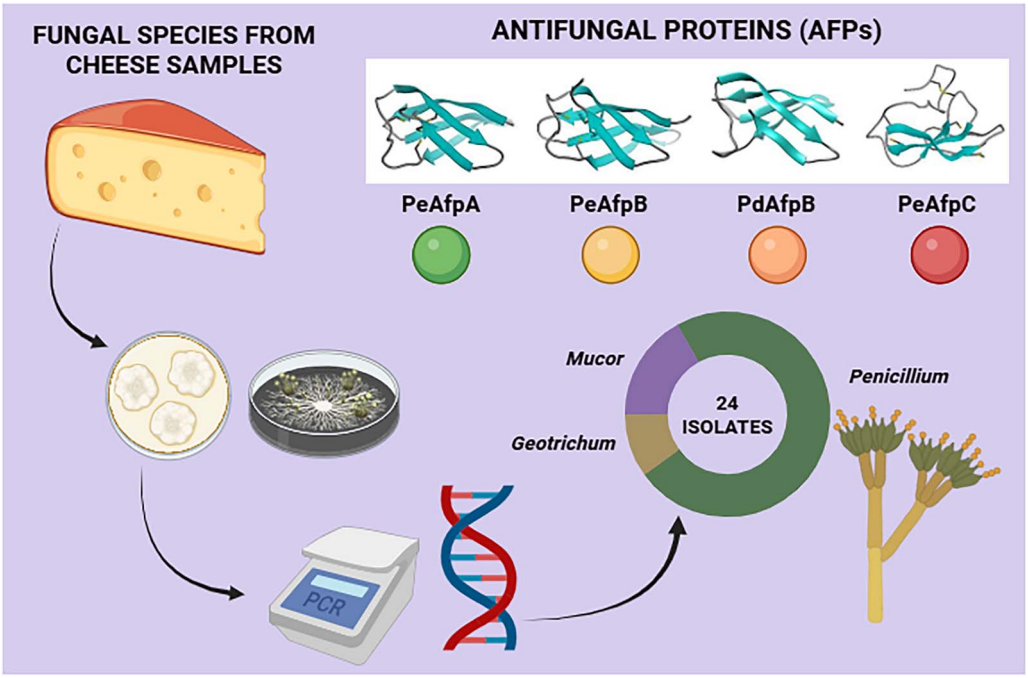
Abstract

This research aimed to isolate and identify fungal species causing cheese spoilage and to assess their susceptibility to various antifungal proteins (AFPs), including PdAfpB from *Penicillium digitatum*, and PeAfpA, PeAfpB, and PeAfpC from *Penicillium expansum*. Twenty-four fungal strains were selected based on morphological criteria and subsequently identified to the species level through sequence analysis. *Penicillium* species, which comprised the majority of the mycobiota, were identified using a combined phylogenetic analysis of ITS, β-tubulin, and Calmodulin sequences, with *Penicillium biforme*, *Penicillium crustosum*, and *Penicillium solitum* being the most prevalent. Additionally, *Geotrichum candidum*, *Mucor circinelloides*, and *Mucor racemosus* were identified. Among the AFPs tested, PeAfpA exhibited the highest potency, completely inhibiting the growth of most tested fungi at concentrations ranging from 1 to 64 μg/ml. PdAfpB demonstrated moderate antifungal activity, followed by PeAfpB, while PeAfpC was the least effective. The research emphasises the promise of these AFPs, especially PeAfpA, as novel antifungal solutions to help prolong cheese shelf life.

Keywords: antifungal proteins, cheese, *Penicillium*, identification, ITS, β-tubulin, calmodulin, spoilage, control

Graphical abstract



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Introduction

Cheese is a globally popular food that provides ideal conditions for fungal growth, owing to its suitable pH and high moisture levels (Motalebi Moghanjougi et al., 2020). The processes of ripening and storage increase its vulnerability to spoilage, leading to substantial financial losses for the cheese industry (Cosentino et al., 2018; Garnier et al., 2017; Muñoz-Tebar et al., 2021). Spoilage rates may climb as high as 50% (Nguyen Van Long et al., 2016) predominantly due to *Penicillium* species, with other contributing genera, including *Aspergillus*, *Cladosporium*, *Alternaria*, *Geotrichum*, *Byssochlamys*, and *Mucor* genera (Garnier et al., 2017). Fungal spoilage results in noticeable changes, including off-flavours that degrade product quality and alter sensory properties. Beyond the unappealing appearance and taste, certain fungi can also produce harmful mycotoxins, posing risks to consumer health (Liu et al., 2020).

Potassium sorbate and natamycin are the most effective preservatives to control fungal contamination in cheese (Garnier et al., 2017; Meena et al., 2021). Sorbates are classified as generally recognized as safe food additives (Schmidt-Heydt et al., 2007), although several negative impacts have been reported, including attention deficit hyperactivity disorder, allergic responses, inflammation-related effects, and potential teratogenic, neurotoxic, and genotoxic outcomes (McCann et al., 2007; Okus et al., 2024; Piper & Piper, 2017; Raposa et al., 2016; Sasaki et al., 2002; Stratford et al., 2007). Moreover, some yeast and fungi have developed the capacity to break down sorbate through decarboxylation, producing trans-1,3-pentadiene, causing an off-flavour and odour described as "kerosene-like" (Casas et al., 2004; Stopforth et al., 2005). Natamycin is a fermentation product from *Streptomyces natalensis* free from odour and colour, which has been used in the cheese industry against fungi and yeasts for more than 40 years. It is a food additive approved by the Food and Drug Administration and the European Union (E235) (Delves-Broughton, 2014; Elsayed et al., 2019); however, in Europe it is only allowed for surface-treatment of hard, semi-hard, and semi-soft cheeses (European Commission Union, 1995). Natamycin can become inactive when exposed to ultraviolet or fluorescent light (Yang, 2010). In consequence, when cheese is exposed to light during transportation and retailing, its preservative effect is reduced (Albini et al., 1998). On the other hand, persistent and extended exposure created a selective pressure that led to the development of natamycin tolerance in fungal strains (Garnier et al., 2017; Streekstra et al., 2016).

In response to consumer preferences for chemical-free food, there is increasing interest in new food preservation methods that utilise natural additives (Lima et al., 2021; Ritota & Manzi, 2020; Silva et al., 2018). Using antifungal proteins (AFPs) for preservation to combat fungal contamination represents an innovative approach to replacing chemical agents in the food industry (Thery et al., 2019). Several features render AFPs interesting in food preservation application (Delgado et al., 2016; Leyva Salas et al., 2017; Shwaiki et al., 2021). They possess a broad-spectrum activity, different mechanisms of action, and stability to pH, high temperatures, and proteolysis (Delgado et al., 2019; Garrigues et al., 2016; Hegedüs & Marx, 2013; Marx et al., 2008). Furthermore, the development of fungal resistance to AFPs is likely to be more difficult due to their multi-step mode of action. Additionally, other functions of AFPs have been reported including the reduction of mycotoxin biosynthesis (Martínez-Culebras et al., 2021b).

In previous studies, AFPs from *Penicillium expansum* (PeAfpA, PeAfpB, and PeAfpC) and *Penicillium digitatum* (PdAfpB) have been characterised and their antifungal activity have been evaluated against a wide panel of fungi from different genera, including human and plant pathogens, mycotoxin-producing fungi, and other species of food interest (Garrigues et al., 2017, 2018; Gandía et al., 2021; Martínez-Culebras et al., 2021a; Valero-Abad et al., 2023; Hernández-García et al., 2024). This study seeks to isolate and identify fungal strains from cheese samples and conduct *in vitro* experiments to assess the antifungal efficacy of these active AFPs against the contaminating mycobiota.

Material and methods

Samples and fungal isolation

Six visually distinct samples, including both cheese and environmental materials, were collected from a cheesemaking facility located in Castilla-La Mancha (Spain) for subsequent fungal isolation in the laboratory. Fungal strains were isolated from hyssop samples and cultured on dichloran rose bengal chloramphenicol medium (Pitt & Hocking, 2009). Colonies with distinct appearances were selected and transferred to malt extract agar (MEA) plates to obtain pure cultures.

Phenotypic identification

Sixty fungal isolates were identified through macroscopic and microscopic observation, with the aid of published guidelines (Frisvad & Samson, 2004; Pitt & Hocking, 2009; Samson et al., 2004; Samson & Frisvad, 2004). For macro-morphological analysis, isolates were inoculated in triplicate on four standardised solid media: potato dextrose agar (PDA), MEA, yeast extract sucrose agar (YES), and czapek yeast extract agar (CYA) (Samson et al., 2004). The cultures were incubated at 28 °C for 7 days in the dark. To determine the micro-morphological characteristics, microscope slides were prepared and examined. Fungal isolates displaying similar macroscopic and microscopic features were grouped together. Representative isolates from each group were then identified at the species level through sequence analysis.

ITS, β-tubulin, and calmodulin gene amplification and sequence analysis

Fungal DNA extraction was performed as described by (Khang et al., 2007). ITS, β-tubulin gene *BenA*, and Calmodulin gene from selected fungal isolates and reference strains of fungi were obtained using the primers its5/its4 (White et al., 1989), Bt2a/Bt2b (Carbone & Kohn, 1999), and CMD5/CF4 (Peterson, 2004; Weir et al., 2012), respectively. The ITS, β-tubulin, and Calmodulin gene sequences of *Penicillium roqueforti* CBS 221.30 (MH855127.1; MN969396.1; HQ442332.1), *Penicillium mononematosum* CBS 172.87 (KC411685.1; AY495997.1; JX996964.1), *Penicillium biforme* CBS 297.48 (KC411731.1; MN969373.1; KU896823.1), *Penicillium crustosum* CV1529 (JX091402.1; JX091538.1; JX141579.1), *Penicillium salamii* ITEM 15291 (NR_156542.1; HG514437.1; HG514432.1), *Penicillium solitum* DRI3 (MZ364044.1; MZ364070.1; MZ364115.1), *Penicillium hordei* CBS 701.68 (MN431391.1; MN969385.1; KU896841.1), *Penicillium paneum* CBS 101032 (NR_103620.1; AY674387.1; HQ442331.1), and *Aspergillus flavus* CBS 100927 (HM560055.1; EF203132.1; EF202063.1) were obtained from the European Molecular Biology Laboratory sequence database. Polymerase chain reaction reactions were conducted in a total volume of 25 μl containing 10 mM Tris–HCl, 50 ng of DNA, 1 μM of each primer, 2 mM of $MgCl_2$, 80 μM (each) dNTPs, and 1 U of DNA polymerase (BIOTAQTM DNA polymerase). The reaction mixtures were subjected to 35 cycles: 30 s at 94 °C, 1 min at 52 °C, and 1 min at 72 °C in a thermocycler (Labnet, MultiGene). The primer pairs CMD5/CF4, Bt2a/Bt2b, and its5/its4 were used to amplify the sequence of both strands of Calmodulin, *BenA*, and ITS sequences, respectively.

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Polymerase chain reaction products were directly sequenced by using the sequencing kit Taq DyeDeoxy (Applied Biosystems, Falmer, Brighton, United Kingdom). Polymerase chain reaction reactions were performed in a final volume of 10 μl, containing 5× reaction buffer (BigDye Terminator), 3.2 μM of each primer, 20–30 ng of DNA, and 1 U of the enzyme PREMISC. The sequencing PCR conditions consisted of 99 cycles: 10 s at 96 °C, 10 s at 52 °C, and 4 min at 60 °C. Polymerase chain reaction products were sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) at the Central Service for Experimental Research (SCSIE) of the University of Valencia. The resulting chromatograms were analysed with MEGA X version 4.1 software (Kumar et al., 2018).

The programme MEGA X version 4.1 (Kumar et al., 2018) was used to align the sequences. Genetic distances were calculated based on the Jukes–Cantor model and the phylogenetic analysis was performed using the neighbour-joining (NJ) method (Saitou & Nei, 1987). The NJ tree and the statistical confidence of specific sequence groups within the tree were assessed using a bootstrap test with 1,000 pseudoreplicates (Hillis & Bull, 1993), all conducted in MEGA X version 4.1 (Kumar et al., 2018).

Antifungal activity assays

Growth inhibition assays were conducted in 96-well microtiter plates (Nunc, Roskilde, Denmark) using the broth microdilution method, as outlined in previous studies (Garrigues et al., 2017; Valero-Abad et al., 2023). Briefly, fungi were grown in a final volume of 100 μl of 5% diluted potato dextrose broth (Scharlau Chemie, S.A., Spain) with 0.01% (wt/vol) chloramphenicol to inhibit bacterial contaminants, containing 5×10^4 conidia/ml and twofold serial concentrations of AFPs ranging from 1 to 64 μg/ml. The three PeAFPs from *P. expansum* and PdAfpB from *P. digitatum* were produced and purified according to earlier protocols (Garrigues et al., 2018; Hernanz-Koers et al., 2018). Plates were statically incubated for 72 hr at 25 °C. Growth was determined every 24 hr by measuring the optical density (OD) at 600 nm using the FLUOstar Omega plate spectrophotometer (BMG Labtech, Orlenberg, Germany). The OD600 mean and *SD* of three replicates were calculated. Dose–response curves were generated from measurements after 72 hr. These experiments were repeated at least twice for each protein-strain combination. The minimal fungicidal concentration (MFC) and minimal inhibitory concentration (MIC) values were determined as described in prior research (Hernández–García et al., 2024; Valero-Abad et al., 2023). A MFC/MIC ratio > 4 was classified fungistatic, whereas a MFC/MIC ratio ≤4 was classified fungicidal (Hazen, 1998; Pfaller et al., 2004).

Results

Fungal isolation from cheese and species identification

A total of 58 fungal strains isolated from cheese were first identified at the genera level using traditional criteria (see *Material and methods*). Predominant mycobiota belonged mainly to the genera *Penicillium* (39 strains, 67.24% of total isolated strains) followed by *Mucor* (14 strains, 24.14%) and *Geotrichum* (5 strains, 8.62%) genera. Based on cultural and morphological comparisons, the 58 strains were categorised into 24 groups. A representative isolate from each group was chosen for species-level identification through sequence-based molecular analysis. Results revealed that the majority of fungi isolated from cheese samples belonged to the *Penicillium* genus, accounting for 17 of the 24 strains. Species-level identification of *Penicillium* isolates was achieved

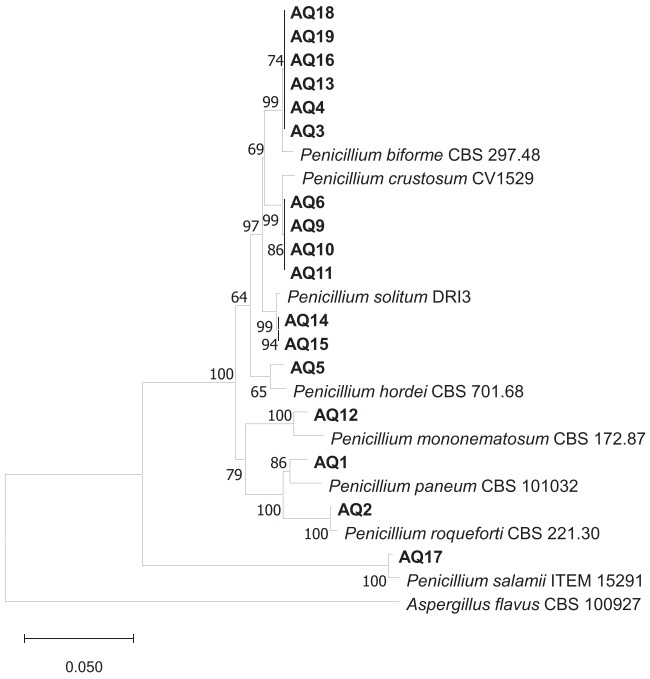

Figure 1. Neighbour-joining tree based on nucleotide divergences, estimated according to Jukes–Cantor model, from the combined datasets of ITS, β-tubulin, and Calmodulin sequences. The numbers on the nodes are the frequency (in percent) with which a cluster appears in a bootstrap test of 1,000 runs.

using a combined phylogenetic approach incorporating ITS, β-tubulin, and Calmodulin gene sequences (Figure 1; Table 1). The phylogenetic analysis divided the *Penicillium spp.* isolates into two clearly distinct clusters (Figure 1). Most of the isolates (16 out of 17) were contained within a well-supported cluster I (bootstrap value, 100). Within this cluster I, isolates AQ3, AQ4, AQ13, AQ16, AQ18, and AQ19 were identified as *P. biforme*; isolates AQ6, AQ9, AQ10, and AQ11 were identified as *P. crustosum*; and isolates AQ14 and AQ15 were identified as *P. solitum*. The remaining isolates were identified as *P. hordei* (AQ5), *P. mononematosum* (AQ12), *P. paneum* (AQ1), and *P. roqueforti* (AQ2). On the other hand, isolate AQ17 was identified as *P. salamii* within a well-supported cluster II (bootstrap value, 100). Overall, *P. biforme* and *P. crustosum* were the most predominant fungal species with 6 (35.3%) and 4 (23.5%) isolates, respectively, followed by *P. solitum* (2 isolates, 14.3%).

Antifungal activity profiles of AFPs

The antifungal activity of the four AFPs (PeAfpA, PeAfpB, PeAfpC, and PdAfpB) was evaluated against the 24 identified fungal strains (Table 1). Variability in antifungal effectiveness was observed among the AFPs (Table 1; Figure 2). Table 1 presents the MIC and MFC values for each protein against all tested fungal species, while Figure 2 illustrates representative dose–response curves comparing their antifungal activities. PeAfpA demonstrated the highest activity, followed by PdAfpB and PeAfpB, which showed comparable effects, and PeAfpC as the least active.

PeAfpA was able to inhibit the growth of almost all tested fungi. MIC values varied from 2 μg/ml against *P. biforme* AQ3 and *P. biforme* AQ4 to 64 μg/ml against *P. paneum* AQ1, *P. roqueforti* AQ2, and *P. hordei* AQ5. Most of the PeAfpA MIC values were in the range of 4–8 μg/ml (Table 1). PdAfpB and PeAfpB also exhibited a broad spectrum of activity and were able to inhibit the growth of a considerable number of fungal species. PdAfpB MIC values ranged from 2 μg/ml against *P. biforme* AQ4 and *P. biforme* AQ19 strains to

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Table 1. Minimal inhibitory concentration (MIC) (μg/ml) and minimal fungicidal concentration (MFC) values of AFPs against cheese isolates.

Fungi	Code	Genbank accession number			PeAfpA			PeAfpB			PeAfpC			PdAfpB		
		ITS	β-tubulin	Calmodulin	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R	MIC	MFC	R
P. paneum	AQ1	PQ426600	PV469306	PV485412	64	>	>	64	>	>	>	>	>	64	64	1
P. roqueforti	AQ2	PQ426601	PV469307	PV485413	64	64	1	64	64	1	>	>	>	32	64	2
P. biforme	AQ3	PQ426602	PV469308	PV485414	2	>	>	2	>	>	>	>	>	4	>	>
P. biforme	AQ4	PQ426603	PV469309	PV485415	2	>	>	8	>	>	>	>	>	2	>	>
P. hordei	AQ5	PQ426604	PV469310	PV485416	64	>	>	32	32	1	>	>	>	32	32	1
P. crustosum	AQ6	PQ426605	PV469311	PV485417	4	4	1	4	4	1	>	>	>	4	4	1
G. candidum	AQ7	PQ426606	–	–	4	4	1	>	>	>	>	>	>	16	>	>
G. candidum	AQ8	PQ426607	–	–	>	>	>	>	>	>	>	>	>	>	>	>
P. crustosum	AQ9	PQ426608	PV469312	PV485418	16	16	1	16	16	1	>	>	>	16	16	1
P. crustosum	AQ10	PQ426609	PV469313	PV485419	8	8	1	16	16	1	>	>	>	32	32	1
P. crustosum	AQ11	PQ426610	PV469314	PV485420	4	8	2	8	8	1	>	>	>	4	4	1
P. mononematosum	AQ12	PQ426611	PV469315	PV485421	4	4	1	>	>	>	>	>	>	32	32	1
P. biforme	AQ13	PQ426612	PV469316	PV485422	4	4	1	>	>	>	>	>	>	32	32	1
P. solitum	AQ14	PQ426613	PV469317	PV485423	4	4	1	64	64	1	>	>	>	8	8	1
P. solitum	AQ15	PQ426614	PV469318	PV485424	4	8	2	64	64	1	>	>	>	8	8	1
P. biforme	AQ16	PQ426615	PV469319	PV485425	16	>	>	>	>	>	>	>	>	>	>	>
P. salamii	AQ17	PQ426616	PV469320	PV485426	8	8	1	64	64	1	>	>	>	32	32	1
P. biforme	AQ18	PQ426617	PV469321	PV485427	8	8	1	64	64	1	>	>	>	8	8	1
P. biforme	AQ19	PQ426618	PV469322	PV485428	8	8	1	32	32	1	>	>	>	2	2	1
M. racemosus	AQ20	PQ426619	–	–	16	>	>	>	>	>	>	>	>	>	>	>
M. racemosus	AQ21	PQ426620	–	–	8	>	>	>	>	>	>	>	>	>	>	>
M. racemosus	AQ22	PQ426621	–	–	16	>	>	>	>	>	>	>	>	>	>	>
M. racemosus	AQ23	PQ426622	–	–	8	8	1	>	>	>	>	>	>	>	>	>
M. circinelloides	AQ24	PQ426623	–	–	16	32	2	>	>	>	>	>	>	>	>	>

Note. 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.

64 μg/ml against *P. paneum* AQ1. PeAfpB MIC values ranged from 2 μg/ml against *P. biforme* AQ3 to 64 μg/ml against *P. paneum* AQ1, *P. roqueforti* AQ2, *P. solitum* AQ14, *P. solitum* AQ15, *P. salamii* AQ17, and *P. biforme* AQ18. Finally, PeAfpC was the least active protein and was not able to completely inhibit the growth of any fungal species at the concentrations tested.

The most susceptible strains to the action of AFPs were *P. biforme* AQ3, *P. biforme* AQ4, and *P. crustosum* AQ6, for which the MIC values of PeAfpA ranged from 2 to 4 μg/ml. On the other hand, the strains more tolerant to AFPs were *Geotrichum candidum* AQ8, *P. biforme* AQ16, *Mucor racemosus* AQ20, *M. racemosus* AQ21, and *M. racemosus* AQ22, as shown in Table 1.

Fungal isolates that exhibited measurable MIC values in liquid medium were further tested to determine their MFC values (Table 1). A certain amount of MFC values could not be determined at the range tested. However, the MFC values obtained for PeAfpA were similar than their respective MIC values. Regarding PdAfpB and PeAfpB, MFC and MIC values were identical (2–64 μg/ml). As MIC and MFC values were largely similar, the resulting MFC/MIC ratios for most fungal isolates ranged from 1 to 2. Based on the conventional criteria established for yeast (Hazen, 1998; Pfaller et al., 2004), all four AFPs demonstrated fungicidal activity against the tested strains, as all MFC/MIC ratios were below 4.

Discussion

Fungal contamination poses a significant problem for the cheese industry, not only because it causes great economic losses but also because some species can produce mycotoxins, which are a major health problem (Buehler et al., 2017; Shehata et al., 2018). In this study, a total of 24 fungi isolated from samples obtained in a cheesemaking were selected based on morphological criteria and subsequently identified at the species level using sequence analysis. Among them, *Penicillium* (17 out of 24) and *Mucor* (5 out of 24) were the most prevalent genera, which are in accordance with the literature (Garnier et al., 2017; Leyva Salas et al., 2018).

The ITS region is widely recognised as the universal genetic barcode, offering enhanced taxonomic resolution across various fungal genera. However, ITS sequences have limitations and are not sufficient for distinguishing closely related *Penicillium* species. In the present study, ITS marker was supplemented with specific markers, such as β-tubulin and Calmodulin genes, for effective identification of *Penicillium* species. Among *Penicillium* species *P. biforme* was the most predominant species isolated followed by *P. crustosum* and *P. solitum*, which is consistent with literature (Anelli et al., 2019; Decontardi et al., 2018; Muñoz-Tebar et al., 2021). It should be pointed out that *P. biforme* was formerly considered synonymous with *P. commune*, but recent studies have redefined it as a distinct species (Giraud et al., 2010; Ropars et al., 2020). *Penicillium biforme* is believed to be a domesticated species derived from the wild *Penicillium fuscoglaucum*, primarily used in cheese production (Ropars et al., 2020), which is considered as safe species for cheeses ageing process. Despite having a functional biosynthetic pathway for ergot alkaloids (EAs), their accumulation has not been reported in cheese for this species. The presence of those EAs was not detected in synthetic medium (CYA) nor in cheese samples naturally contaminated by *P. biforme* (Anelli et al., 2024). *Penicillium solitum* is also considered to pose no proven toxigenic risk to guide cheese ageing (Wu et al., 2019). Other *Penicillium* species commonly isolated in cheese are producers of mycotoxins: *P. crustosum* (penitrem, roquefortine) and *P. roqueforti* (isofumigaclavins A and B, mycophenolic acid, roquefortine, PR-toxin) (Dorner et al., 1980; Otero et al., 2020; Polonelli et al., 1987). On the other hand, some *Penicillium* species identified in this study are not normally isolated from cheese. *Penicillium paneum*

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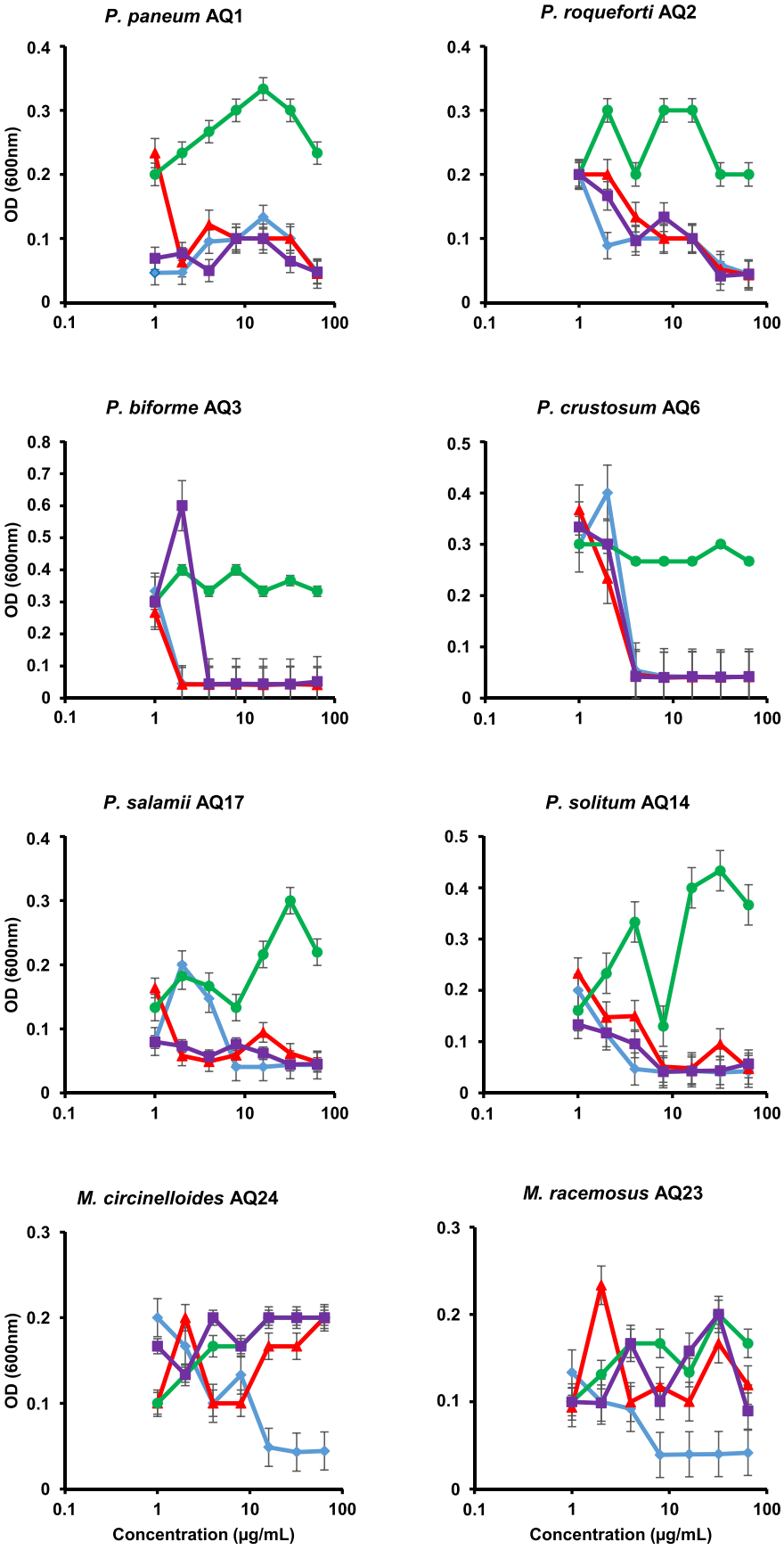

Figure 2. Dose–response curves comparing the antifungal activity of PeAfpA (blue diamonds), PeAfpB (red triangles), PeAfpC (green circles), and PdAfpB (purple squares) against bread spoilage fungi. Dose–response curves show mean OD600±SD of three technical replicates from each of two independent biological replicates, measured after 72 hr at 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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usually affects bread (Garcia et al., 2019; Nielsen et al., 2006), *P. mononematosum* has been found on leaves, berries, grape juice, and tomato (Kamil et al., 2021; Regecová et al., 2022), *P. salamii* is commonly used in dry-cured meat (Lo et al., 2023), and *P. hordei* has been isolated from cereal grains (Frisvad & Samson, 2004). These *Penicillum* species can also produce some mycotoxins, such as patulin and roquefortine C (Perrone & Susca, 2017; Zhao et al., 2024), but due to their low frequency of isolation it appears not to be a significant problem of mycotoxin contamination in cheese. There are no specific regulations in the European Union for mycotoxins in cheese, except for aflatoxin M1, which is a hydroxylated derivative of aflatoxin B1 commonly found in milk and dairy products due to its excretion by lactating animals. Due to the limited data available in the literature regarding the presence of OTA in cheese, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM Panel) emphasised the need for more data on this subject (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2020). In the present study, no OTA-producing fungi, such as *Penicillium verrucosum* or *Penicillium nordicum*, were isolated.

The remaining isolated species of the study belonged to the genera *Mucor* and *Geotrichum*, typically related to the cheese industry. *Mucor racemosus* is one of the most commonly found species in cheese samples (De Respinis et al., 2023; Lebreton et al., 2020) and *M. circinelloides*, although less frequent, has been used in previous studies in the production of fresh cheese (Bensmail et al., 2023). It is important to point out that the genus *Mucor* can be harmful, as it can infect immunocompromised patients causing mycormycosis (Okiki et al., 2011). Finally, *G. candidum* is a fungus that promotes the development of flavours and aromas in cheese, so its presence in this type pf product is expected (Kamilari et al., 2023).

Antifungal proteins were effective at the concentrations tested (1–64 μg/ml) against the 23 out of the 24 fungal isolates, though their efficacy varied notably between proteins. These differences in protein activity may be due to their differences in sequence motifs, length, cationicity, disulphide bond patterns, and number of cysteine residues (Garrigues et al., 2017, 2018; Giner-Llorca et al., 2023). In addition, it has been observed in this study that, not only are there differences in fungal susceptibility to proteins among fungal species, but there are also differences between what could be different strains of the same species (Table 1). Studies using more strains of the same species would be necessary to determine more aspects of fungal susceptibility and resistance to the proteins used.

Among the AFPs evaluated, PeAfpA showed the highest antifungal activity, followed by proteins belonging to class B (PdAfpB and PeAfpB). However, it has been observed that depending on the fungal species that PdAfpB faces, its activity is more like PeAfpA or PeAfpB. On the other hand, PeAfpC, unlike the other more active proteins, showed no antifungal activity. This lack of activity may be explained by differences in its physicochemical characteristics, particularly its lower isoelectric point (pI 6.87). Antifungal proteins with higher positive charges at neutral pH, such as PeAfpA (pI 9.47) and PdAfpB (pI 9.06), tend to exhibit stronger interactions with negatively charged fungal membranes, which is considered a crucial step in their mode of action. The relatively lower net positive charge of PeAfpC could therefore result in reduced membrane binding and, consequently, limited antifungal efficacy (Garrigues et al., 2018). Moreover, previous studies have highlighted that PeAfpC displays selective antifungal activity, showing effectiveness mainly against *Byssochlamys spectabilis* species. This type of specificity is also seen in other class C AFPs, reinforcing the idea that sequence or structural features unique to this class may dictate a more restricted range of activity (Martínez-Culebras et al., 2021a; Valero-Abad et al., 2023). The discrepancies in protein activity observed across studies may be attributed to the use of different fungal species, reinforcing the idea that antifungal activity is species-specific.

Results indicate that the AFPs evaluated in this study may exhibit a fungicidal effect, which is an advantage compared to the fungistatic effect of chemical preservatives used in the cheese industry that require high concentrations to control fungal growth. Apart from the adverse effects previously indicated (Okus et al., 2024; Piper & Piper, 2017; Raposa et al., 2016), the use of high doses of synthetic preservatives can negatively impact the organoleptic properties of cheese, and repetitive treatments may lead to the development of resistant strains, as observed with *P. roqueforti* (Garcia et al., 2021). On the contrary, the use of potassium sorbate in concentrations lower than those recommended can increase the production of mycotoxins, as occurred in the case of *Aspergillus carbonarius*, where the production of ochratoxin A increased (Fodil et al., 2018). Similarly, continuous and prolonged selection pressure led to the development of natamycin tolerance in fungal strains (Garnier et al., 2017; Streekstra et al., 2016). Therefore, the use of AFPs with a fungicidal effect could be a good solution for controlling fungal spoilage and preventing mycotoxin contamination. Furthermore, a previous study (Hernández-García et al., 2024) compared the antifungal activity of PeAfpA with that of commonly used fungicides, such as imazalil and thiabendazole, and preservatives, including natamycin, calcium propionate, sodium benzoate, and potassium sorbate. The results showed that, overall, PeAfpA exhibited stronger antifungal activity than the other compounds tested. Additionally, the same work states that AFPs, in particular PeAfpA, could be used in combination with natamycin, with which a synergistic effect has been recently demonstrated. However, more comprehensive studies are needed to evaluate the fungicidal or fungistatic effect of AFPs.

Interest in using antimicrobial peptides and proteins for cheese preservation has grown in recent years, in particular, bacteriocins and bacteriocin-producing lactic acid bacteria (Ceren Akal & Ozturkoglu-Budak, 2022; Moula Ali et al., 2022). Most studies have evaluated the efficacy of these preservative agents by adding them directly to raw milk before fermentation (Cosentino et al., 2018; Zhang et al., 2024). However, their application on the cheese surface by coating or through packaging films has also improved preservation efficiency (Garnier et al., 2019; Zheng et al., 2022). Additionally, other proteins and peptides from different origins have also been successfully used to preserve cheese. They include dairy-derived bioactive peptides (Öztürk et al., 2022) and antifungal peptides derived from kenaf seed (Arulrajah et al., 2023). To date, no studies have evaluated the use of purified fungal AFPs as preservatives for extending the shelf life of cheese products.

The successful use of AFPs as food preservatives also depends on their safety, production viability, and cost-effectiveness. Regarding toxicity, PeAfpA, PeAfpB, and PeAfpC have shown no haemolytic activity *in vitro* at concentrations up to 100 μM, indicating a lack of cytotoxicity against eukaryotic cells (Garrigues et al., 2018). In terms of manufacturing, the efficient production of PdAfpB, as shown by Gandía et al. (2022), makes it possible to obtain a stable, pure, and active protein in enough quantity for practical use. Furthermore, their high antifungal efficacy at low concentrations (1–64 μg/ml) suggests that small quantities may be sufficient for preservation, making their use potentially cost-effective. However, further studies will be needed to better understand the costs and benefits of producing AFPs on a larger scale and using them in food preservation. In conclusion,

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the genus *Penicillium* represented most of the contaminant mycobiota of cheese. Precise identification of fungal species using a combined phylogenetic analysis of ITS, β-tubulin, and Calmodulin sequences showed a large diversity of *Penicillium* species, with *P. biforme* being the most abundant. Considering the potent and the broad *in vitro* inhibition spectrum of the AFPs tested, in particular PeAfpA, it seems likely that this protein might also be effective in extending the shelf life of cheese products. Nevertheless, the antifungal effectiveness of PeAfpA still requires in situ evaluation. Future efforts will be directed to study different PeAfpA application methods to extend the shelf-life of cheese products.

Conclusion

This study highlights the diversity of fungal species contaminating cheese, with a predominance of *Penicillium spp.*, particularly *P. biforme*, *P. crustosum*, and *P. solitum*. Molecular identification using ITS, β-tubulin, and Calmodulin sequences proved essential for accurate species differentiation within this complex genus. Importantly, the AFPs PeAfpA, PdAfpB, and PeAfpB demonstrated promising activity against most isolates, with fungicidal effects confirmed by low MFC/MIC ratios. PeAfpA stood out as the most effective, inhibiting a broad range of cheese-associated fungi. These findings support the potential of AFPs as natural biopreservatives to combat fungal spoilage in the dairy industry, offering an alternative to traditional chemical preservatives. Future work should focus on the application of these AFPs in real cheese matrices to validate their efficacy under commercial storage and ripening conditions.

Data availability

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Author contributions

Laura Hernández-García (Data curation, Formal analysis, Investigation, Methodology, Writing—original draft), Patricia Roig (Conceptualisation, Visualisation, Writing—review & editing), Pedro V. Martínez-Culebras (Conceptualisation, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Visualisation, Writing—review & editing), and Xabier Molinos (Data curation, Formal analysis, Investigation, Methodology, Writing—original draft)

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Conflicts of interest

The authors declare no conflict of interest.

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