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Proteomic profiling and glycomic analysis of the yeast cell wall in strains with Aflatoxin B_1 elimination ability

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Summary

The use of microorganisms for Aflatoxin B_1 elimination has been studied as a new alternative tool and it is known that cell wall carried out a critical role. For that reason, cell wall and soluble intracellular fraction of eight yeasts with AFB_1 detoxification capability were analysed. The quantitative and qualitative comparative label-free proteomic allowed the identification of diverse common constituent proteins, which revealed that putative cell wall proteins entailed less than 10% of the total proteome. It was possible to characterize different enzymes linked to cell wall polysaccharides biosynthesis as well as other proteins related with the cell wall organization and regulation. Additionally, the concentration of the principal polysaccharides was determined which permitted us to observe that β-glucans concentration was higher than mannans in most of the samples. In order to better understand the biosorption role of the cell wall against the AFB_1, an antimycotic (Caspofungin) was used to damage the cell wall structure. This assay allowed the observation of an effect on the normal growth of those yeasts with damaged cell walls that were exposed to AFB_1. This effect was not observed in yeast with intact cell walls, which may reveal a protective role of this structure against mycotoxins.

Introduction

Mycotoxins are toxic secondary metabolites produced by some mould species from genera *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria* (Bennett and Klich, 2003). Different groups of mycotoxins have been associated with food and feed such as aflatoxins, ochratoxin, fumonisins, zearalenone, patulin and trichothecenes (Bennett and Klich, 2003). Occurrence of mycotoxins in feeds and foods depends on the growth of the fungi before or after harvest as well as some favourable conditions environments, high temperatures and humidity among other external factors (Tola and Kebede, 2016). One of the most dangerous are aflatoxins synthesized by *Aspergillus flavus* and *parasiticus*, which include AFB_1, AFB_2, AFG_1, AFG_2 or AFM_1. Of these, AFB_1 is the most carcinogenic and, also, it has been reported to be a causative agent of a growth suppression, immune system modulation, malnutrition and hepatocellular carcinoma (Rushing and Selim, 2019).

Some methodologies have been developed with the aim of reducing or removing aflatoxins from different food substrates, such as the use of electromagnetic radiation (Gamma or ultraviolet radiation), ozone fumigation and treatment with chemical control agents (Udomkun *et al*., 2017). However, chemical and physical detoxification methods have some negative effects on the nutritional value and organoleptic characteristics of the food matrix, and in addition the requirement for complex and high-cost equipment that make some of these methods prohibitive to put in practice (McCormick, 2013). Microbiological strategies are a promising alternative for removing these metabolites from food and feeds, either through biosynthesis of extracellular enzymes or by adsorption on microbiological cell walls (Fruhauf *et al*., 2012; McCormick, 2013).

Previous investigations have demonstrated the potential use of yeasts to remove mycotoxins, either by live cells, cell walls or cell wall extracts. It is estimated that the yeast cell wall represents 26%–32% of the dry weight of cells and its structure is dynamic and adaptable to physiological and morphological changes (Aguilar-Uscanga and François, 2003). Polysaccharides constitute 75% of the cell wall dry weight and include mannose, β-glucans (mainly (1 → 3)-β-D-glucan) and polymers of N-acetylglucosamine or chitin (Aguilar-Uscanga and François, 2003). Proteins from the yeast cell wall are mainly associated with D-mannose, forming the mannoproteins complex that is comprised of glycosylphosphatidylinositol (GPI)-modified proteins and alkali-sensitive linkage proteins

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that play an important role in adhesion to other cells, biofilm formation and cell wall biogenesis. In addition, extracellular enzymes associated with cell wall polysaccharides biosynthesis or cell wall protein maintenance have been described in *C. albicans* and in *S. cerevisiae* (Hsu *et al*., 2015).

The adsorption capability of yeast cell wall to protect against mycotoxins has been previously reported (Fruhauf *et al*., 2012; Petruzzi *et al*., 2014; Piotrowska and Masek, 2015), however, studies have been mainly focused on the model yeast *S. cerevisiae*, and neither cell wall proteins or polysaccharides, which may be involved in the adsorption mechanism, are well described for most of the non-*Saccharomyces* species.

For these reasons, the aim of this research is to study the composition of cell wall proteins and carbohydrates from yeasts that showed a detoxification ability against AFB_1 in previous studies, as well as, to study the role of the cell wall in protection against AFB_1 toxicity.

Material and methods

Yeast cultures

Strains from *Saccharomyces* and non-*Saccharomyces* genera were chosen owing to their proven AFB_1 detoxification capability (García-Béjar *et al*., 2020a). They were environmental isolates of *Aureobasidium pullulans* (FH1), *Candida parapsilosis* (ECF42), *Wickerhamiella sorbophila* (ECF12; formerly *Candida sorbophila*), *Candida tropicalis* (AK11), *Diutina rugosa* (FR19 and ECF61), *Rhodotorula mucilaginosa* (EB39) and *Saccharomyces cerevisiae* (EB83). All these strains were catalogued and are kept in the University of Castilla – La Mancha (UCLM) yeast collection (García-Béjar *et al*., 2020b).

Proteomic response in the presence of Aflatoxin B_1

One representative species, *S. cerevisiae* (EB83) was selected to determine the proteomic response of yeast to AFB_1. This species was grown in presence of different concentrations of AFB_1 for 24 h in aerobic conditions and was harvest when the stationary phase was reached.

The yeasts from each species were grown in triplicate in 3 ml of YPD broth (24 h, 30°C and 150 rpm; yeast extract 10 g L^{-1}, peptone 20 g L^{-1} and glucose 20 g L^{-1}) in two batches ('Control' or 'Treated sample'). The cultures were centrifuged (3000 rpm/5 min at 4°C) and the pellets were washed with sterile dH_2O and added to 25 ml of MSM + Glu (Glucose 10 g, K_2HPO_4 0.4 g, KH_2PO_4 0.2 g, NaCl 0.1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $MnCl_2$ 0.01 g, $Fe(SO_4)_3$ 0.01 g and Na_2MoO_4 0.01 g L^{-1}; pH 7) in 'Controls' and 25 ml of MSM + Glu + 3 mg L^{-1} of AFB_1 in the case of the 'Treated samples'. These analyses were also carried out in duplicate for 0.04, 1 and 2 mg L^{-1} AFB_1. All samples were incubated for 24 h at 30°C in agitation (150 rpm).

After the incubation time, pellets were collected by centrifugation (3000 rpm/5 min at 4°C), washed with ice-cold phosphate-buffered saline (PBS) and centrifuged again under the same conditions. The pellets were then frozen with liquid nitrogen and all samples were kept at −80°C until the next step. Protein extraction and analysis were carried out as described below.

Proteomic analysis

Growth conditions and protein extraction. The yeast strains were inoculated in 25 ml YPD broth by triplicate for 24 h at 30°C in aerobic conditions and cells were centrifuged when stationary phase was reached (1000*g*/5 min/4°C). Pellets were washed twice with ice-cold PBS and centrifuged again in the same conditions. Samples were then frozen with liquid nitrogen and kept at −80°C until extraction.

Proteins were extracted following an adaptation of the protocol used by Owens *et al*. (2015). Frozen pellets were resuspended in a lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 20 mM EDTA, 10% Glycerol, 1 mM PMSF, 1 μg ml^{-1} Pepstatin A, pH 7.0) and sonicated three times for 10 s at 20% of power followed by incubation on ice for 1 h. Samples were not clarified prior to digestion, to retain cell-wall associated proteins in the mixture. Protein concentration was adjusted to 0.25 mg ml^{-1} with 50 mM ammonium bicarbonate and proteins (100 μl) were reduced using 0.5 M DTT (1 μl) for 20 min at 56°C. Samples were cooled to room temperature and alkylated using 0.55 M IAA (2.7 μl), in the dark for 15 min. Proteins were digested overnight at 37°C in the presence of ProteaseMax (Promega) (1% (wt./vol.); 1 μl) and sequencing grade trypsin (1 μg $μl^{-1}$; 1 μl), and digestion was stopped by addition of 1 μl of trifluoroacetic acid. Acidified samples were centrifuged 12 000*g* for 10 min at room temperature and the supernatants (tryptic peptides) were dried in a SpeedyVac at 30°C for 3 h. Then, samples were cleaned up using C18 Zip-Tips (Thermo fisher), dried again for 1 h and kept in the −20°C freezer until mass spectrometry-based proteomic analysis.

Q-Exactive mass spectrometer. Label-free quantitative proteomics was carried out using a Dionex UltiMate 3000 RSLCnano coupled to a Thermo Q-Exactive mass spectrometer as outlined previously (Collins *et al*., 2017). Peptide mixtures (0.75 μg) were separated on a PepMap EasySpray C18 column (500 mm × 75 μm; 2 μm particle size) using a 2 h gradient from 4% to 35% B (A: 0.1% formic acid (FA), B: 0.1% FA in 80% acetonitrile). MS/MS was performed using a Top15 method at 70 000 resolution for MS scans.

Bioinformatic analysis. Generated data were analysed using MaxQuant software (Version 1.6.2.10) against relevant protein databases with the LFQ algorithm utilized (Cox *et al*., 2014). The databases were downloaded from http://www.uniprot.org (January 2019) or were obtained from https://www.ncbi.nlm.nih.gov/ (*D. rugosa*, November 2019). When a protein database was not available for a species, the database of genetically closely related species was used. Additionally, Perseus software (Version 1.6.2.3) was utilized to organize these data. Proteins that matched to a contaminant database or the reverse database were eliminated. For the final analysis, only proteins detected in at least two replicates were selected (Owens *et al*., 2015).

Blast2GO software was used for the functional analysis of proteins (Gotz *et al*., 2008). In order to identify those proteins that constituted the cell wall or were related with cell wall biosynthesis or organization, protein IDs obtained with Blast2Go software were filtered using the following gene ontology (GO) terms: Cell wall organization, fungal-type cell wall organization, fungal-type cell wall organization or biogenesis, structural constituent of cell wall, cell wall, fungal-type cell wall or yeast-form cell wall which included biological functions and cellular component terms. The proteins associated with these terms were classified based on whether they were constituent, were polysaccharide biosynthesis enzymes or had other roles in regulation of the cell wall.

Quantification of the cell wall principal polysaccharides

Obtaining fungal biomass. Yeast strains were inoculated in 25 ml YPD broth by triplicate and incubated for 24 h at 30°C. Biomass was recovered by centrifugation (1000*g*/5 min/4°C), washed with ice-cold PBS and dried at 60°C. Finally, the samples were milled using a Dounce homogenizer and stored at room temperature in dark and dry conditions.

β-Glucans and D-mannose assays. For β-glucans analysis, these polysaccharides were obtained using an alkali solution (KOH) and were measured by an enzymatic kit (Megazyme©) that allowed their quantification by absorbance measurements (510 nm). Additionally, positive and negative controls (yeast β-glucan and starch preparations respectively), as well as glucose standard and blanks (sodium acetate buffer) were analysed.

D-mannose quantification was carried out by using a D-mannose, D-fructose and D-glucose enzymatic kit (Megazyme) by spectrophotometric measurements (340 nm). Results were obtained using a glucose standard.

Study of the cell wall protective effect against Aflatoxin B$_1$. In an effort to better understand the protective effect of the cell wall in yeasts, an antimycotic compound (Caspofungin) was used to damage its structure. Caspofungin-treated yeast cells were incubated in the presence of AFB$_1$ and cell behaviour was monitored by growth curves.

Minimal inhibitory concentration determination for Caspofungin. For determining the minimal inhibitory concentration (MIC) for each strain, yeast strains were grown in 5 ml of YPD broth at 30°C for 24 h and 150 rpm and a lawn was spread on the surface of YPD agar plates using a sterile swab. Different concentrations of Caspofungin (0.1, 0.25, 0.5, 1 and 2 μg ml^{-1}) were then spotted onto the plate surface (20 μl). Plates were incubated for 24 h at 30°C. The MIC was established as the first concentration at which an inhibition halo was detected for each strain.

Aflatoxin B$_1$ toxicity test. Once the MIC for each strain was established, a toxicity test was carried out to ascertain the effect of AFB$_1$ on the Caspofungin-damaged cell.

The pellets from young cultures were collected by centrifugation (1000*g*/5 min/4°C) and washed twice with sterile dH_2O. Cell concentration was adjusted to 10^6 cells ml^{-1} and inoculated in MSM + Glu (Glucose 10 g, K_2HPO_4 0.4 g, KH_2PO_4 0.2 g, NaCl 0.1 g, $MgSO4{\cdot}7H_2O$ 0.5 g, $MnCl_2$ 0.01 g, $Fe(SO_4)_3$ 0.01 g and Na_2MoO_4 0.01 g L^{-1}) for a final volume of 200 μl with different conditions: Yeast control (MSM + Glu), Caspofungin control (MSM + Glu + MIC Caspofungin), AFB$_1$ control (MSM + Glu + 0.04 mg L^{-1} AFB$_1$) and Treated sample (MSM + Glu + MIC Caspofungin +0.04 mg L^{-1} AFB$_1$).

Analysis was carried out in triplicate and kinetic monitoring (HiPo MPP-96, Biosan) by absorbance at 620 nm was read for 24 h at 30°C. Measurements were taken every 15 min with an agitation of 5 s before reading. Kinetic growth curves were obtained by plotting the optical density (OD) at 620 nm versus time.

Results and discussion

Proteomic response in the presence of Aflatoxin B$_1$

The response of the proteome of EB83 (*S. cerevisiae*) strain in the absence and the presence of AFB$_1$ (0.04, 1, 2 and 3 mg L^{-1}) was analysed by label-free mass spectrometry–based proteomics. A principal component analyses revealed no distinction between untreated samples and those treated with AFB$_1$ (0.04, 1, 2 or 3 mg L^{-1}) (Supplementary Fig. S1). This was also demonstrated using hierarchical clustering, where the proteome of AFB$_1$-treated samples did not cluster distinctly from the untreated samples, for any of the tested concentrations of AFB$_1$ (Supplementary Fig. S2).

Exposure of *S. cerevisiae* to a range of concentrations of AFB_1 showed limited proteomic remodelling as determined by label-free quantitative analysis. Treatment with 0.04 mg L^{-1} AFB_1 did not produce any statistically significant proteome changes above the fold change threshold ($p < 0.05$, fold change ≥2). Higher concentrations of AFB_1 still produced a muted proteomic response, with 1 mg L^{-1} AFB_1 resulting in only six proteins with altered abundance and no changes noted following treatment with 2 mg L^{-1} AFB_1 ($p < 0.05$, fold change ≥2) compared to the untreated control. These limited changes to the proteome indicate that AFB_1 does not induce proteomic remodelling in *S. cerevisiae* EB83. The ability of this strain to remove aflatoxin from the environment instead may be facilitated by biosorption via the cell wall, preventing entry to the cell, and thus limiting any substantial molecular response. In contrast, AFB_1 provoked a large number of changes to the proteome of human hepatocytes, even as assessed using a lower sensitivity gel-based proteomic approach, highlighting the potential system-wide effects that can be produced in eukaryotic cells upon AFB_1 exposure (Zhu *et al*., 2020).

It is known that adsorption is the main mechanism reported by other authors (Piotrowska and Masek, 2015) for mycotoxin elimination and it is produced by non-covalent interactions with the yeast cell wall (Ringot *et al*., 2007). On the other hand, a previous study indicates that AFB_1 was not absorbed or metabolized by the yeast cells which could support the hypothesis that mycotoxins could be removed by biosorption to the cell wall (García-Béjar *et al*., 2020a).

Proteomic analysis

In-solution tryptic digestion of proteins from yeast lysates, including cell wall material, was carried out and qualitative proteomic analysis was performed using liquid chromatography-tandem mass spectrometer. Bioinformatic analyses were conducted to identify putative cell wall proteins as well as those involved in the cell wall organization or biogenesis of the yeasts. The total number of proteins identified was different for each strain, even those that shared the same protein database (i.e. *D. rugosa*).

Cell wall–related proteins constituted no more than 10% of the total detected proteome (Table 1), although in the case of *A. pullulans* (FH1) and *Rh. mucilaginosa* (EB83) these proteins accounted for less than 1% of the detected proteome, while *C. tropicalis* (AK11) and *C. parapsilosis* (ECF42) showed the highest values with a total of 171 (7.8%) and 155 proteins (6.6%) respectively. From the model yeast *S. cerevisiae* (EB83), a total of 88 of the identified proteins (4.1%) were catalogued as cell wall–related proteins. The number of detected cell wall proteins does not appear to positively relate to a better detoxification capability, for example, the strains in which the lowest number of cell wall-associated proteins were detected, showed the highest AFB_1 elimination percentage (*A. pullulans* – FH1; *Rh. mucilaginosa* – EB39) (García-Béjar *et al*., 2020a). Therefore, the effect on mycotoxin adsorption may be more related with the type and concentration of specific proteins rather than the number of proteins. Additionally, it is known that polysaccharides also play an important role in the process of biosorption (Yiannikouris *et al*., 2006; Fochesato *et al*., 2020).

Table 1. Quantification of the total proteome and cell wall proteins of the studied strains.

Species (Strain code)	Total proteins	Cell wall proteins (% of the total proteome)
A. pullulans (FH1)	2838	23 (0.8%)
C. parapsilosis (ECF42)	2333	155 (6.6%)
C. tropicalis (AK11)	2199	171 (7.8%)
D. rugosa (FR19)	2284	89 (3.9%)
D. rugosa (ECF61)	2186	73 (3.3%)
Rh. mucilaginosa (EB39)	2272	19 (0.8%)
S. cerevisiae (EB83)	2168	88 (4.1%)
W. sorbophila (ECF12)	2439	46 (1.9%)

Other proteomic studies of yeast cell wall have been mainly focused on *S. cerevisiae* and *C. albicans* strains. Some successfully identified around 25 cell wall proteins from *S. cerevisiae* only describe bound proteins or those associated with cell wall carbohydrate biosynthesis (Hsu *et al*., 2015). In this study, from *S. cerevisiae* EB83 strain a higher number of proteins associated with the cell wall organization were described which may be related with the analysis conditions applied. Likewise, Champer *et al*. (2016) found fewer cell wall proteins for *C. parapsilosis* (78) and *C. tropicalis* (51) compared with this study, although the total number of proteins detected was also smaller (<700). Proteomic profiles of *A. pullulans* and *Rh. mucilaginosa* have been analysed under certain external factors (Sheng *et al*., 2014; Ilyas *et al*., 2016), however, the number of cell wall–related proteins were not quantified for those cases. In the case of *D. rugosa* and *W. sorbophila*, a previous study analysed the zinc-associated changes to the proteome (García-Béjar *et al*., 2020c); however, no specific analysis for the cell wall–related proteins was carried out.

Cell wall organization proteins and enzymes involved in biosynthesis

Qualitative analysis carried out using Blast2GO allowed the cataloguing of the different cell wall proteins depending on their GO terms generated.

Protein constituents of the cell wall

Among all the proteins identified, some of them were catalogued as structural constituents of the cell wall

Table 2. Cell wall constituent proteins, mannoproteins and glycosylphosphatidylinositol-anchored proteins identified in the different yeast strains analysed.

Protein ID	Strain	Protein name
Cell wall constituent proteins		
A0A074YPF8	*A. pullulans* (FH1)	Related to stress response protein
A0A074X7U7; G8BHR6	FH1; *C. parapsilosis* (ECF42)	Cell wall integrity protein SCW1
A0A074X4N7	FH1	Cell wall galactomanno protein
G8BAM6	ECF42	PIR1-1,3-β-glucan-linked structural cell wall protein
G8B797		Cell wall protein
G8B966		Ssr1 β-glucan associated ser/thr rich cell wall protein
A0A2T0FFB7; O13547	*W. sorbophila* (EFC12); *S. cerevisiae* (EB83)	Covalently linked cell wall protein 14
A0A2T0FKH2	EFC12	Cell wall SED1
A0A2T0FKP5		Hydrophobic surface binding protein A
A0A2T0FPR6; P38248	EFC12; EB83	Cell wall protein ECM33
C5M4X4	*C. tropicalis* (AK11)	Protein PIR1 precursor
C5MD92		Cell wall acid trehalase ATC1
C5MAN3		Cell wall protein 1
KAA8907696.1	*D. rugosa* (FR19; ECF61)	DEHA2A05280p
A0A109FEW3	*Rh. mucilaginosa* (EB39)	Cell wall integrity signalling protein
P40442; Q05164	EB83	Putative uncharacterized protein YIL169C
P46992		Cell wall protein YJL171C
P28319		Cell wall CWP1
Cell wall mannoproteins		
A0A074XJ70; P32478	FH1; EB83	Cell wall protein PIR5
G8BJX3; C5M5B5	ECF42; AK11	Mp65 cell surface mannoprotein
Q03178	EB83	Cell wall mannoprotein PIR1
Q03180		Cell wall mannoprotein PIR3
P47001		Cell wall mannoprotein CIS3
Q12355		Cell wall mannoprotein PST1
Glycosylphosphatidylinositol (GPI) proteins and related to GPI protein/anchor metabolism		
G8B8N3; A0A367XRQ9; KAA8903457.1	ECF42; AK11; FR19; ECF61	GPI-anchored extracellular protein putative
G8BGU6	ECF42	Utr2 GPI-anchored cell wall putative glycoside
G8BID5; C5M2R3; Q03674	ECF42; AK11; EB83	GPI-anchored protein of cell wall
G8BCF3; P36051; KAA8906216.1	ECF42; EB83; FR19	GPI-ethanolamine phosphate transferase
G8B692; C5MJA2	ECF42; AK11	Glycolipid-anchored surface protein
C5MCP6	ECF42	Dolichol-phosphate mannosyltransferase
C5MIX2	AK11	Uncharacterised GPI-anchored cell wall protein
C5M4G8; KAA8907589.1	AK11; FR19; ECF61	Protein CWH43
A0A109FCL2	EB39	Cell wall surface anchor family protein

(Table 2) which were identified in all strains. FR19 and ECF61 (*D. rugosa*) were the strains with the smallest number (4 and 3 respectively), while *S. cerevisiae* (EB83) showed the highest number of structural cell wall proteins (12). This could possibly be as a result of its proteome being more deeply described as a model organism; however, the Blast2GO analysis compares the detected proteins from each strain to the wider fungal database, expanding the annotations for the less characterized strains. Additionally, a total of 18 GPI-anchored proteins were detected across the tested strains, with none detected in *A. pullulans* (FH1).

The analysis detected some common proteins across the species, although strain-specific cell wall proteins were also identified from the proteomes (Table 2). This was the case for PIR5 (A0A074XJ70; P32478), detected in *S. cerevisiae* (EB83) and *A. pullulans* (FH1) strains, as well the covalently linked cell wall protein 14 CCW14 (A0A2T0FFB7; O13547) and cell wall protein ECM33 (A0A2T0FPR6; P38248), which were identified in *S. cerevisiae* (EB83) and *W. sorbophila* (ECF12). Additionally, other proteins were shared by different non-*Saccharomyces* strains tested such as Cell wall integrity protein SCW1 (A0A074X7U7; G8BHR6), which was identified in *A. pullulans* (FH1) and *C. parapsilosis* (ECF42), and Mp65 cell surface mannoprotein (G8BJX3; C5M5B5), which was detected both in *C. tropicalis* (AK11) and *W. sorbophila* (ECF12).

Most of the cell wall–related proteins identified are predicted to be involved in cell wall integrity. Analysis has also revealed the presence of proteins linked to carbohydrates like mannans (Q03178; Q03180; P47001; Q12355; G8BJX3; C5M5B5), galactomannans (A0A074X4N7) and 1,3-β-glucans (G8BAM6; G8B966). Mannoproteins have

been mainly detected in *S. cerevisiae* (EB83), *C. parapsilosis* (ECF42) and *C. tropicalis* (AK11) strains, while galactomannoprotein and β-glucan-linked proteins have only been detected in *A. pullulans* (FH1) and *C. parapsilosis* (ECF42) strains respectively (Table 2).

Among the GPI proteins, and those related to GPI metabolism, were the GPI-anchored extracellular protein (G8B8N3; A0A367XRQ9; KAA8903457.1), GPI-anchored protein of cell wall (G8BID5; C5M2R3; Q03674), GPI-ethanolamine phosphate transferase (C5MIX2; KAA8906216.1), glycolipid-anchored surface protein (G8B692; C5MJA2) and protein CWH43 (C5M4G8; KAA8907589.1), which were detected in multiple strains, except *A. pullulans* (FH1) and *Rh. mucilaginosa* (EB39), as is shown in Table 2. It is interesting to point that strains from *Candida* genus also shared cell wall and GPI proteins that were characterized mainly in *C. parapsilosis* (ECF42) and *C. tropicalis* (AK11) (Table 2).

Mannoproteins are the second most important component of yeast cell wall and can belong to PIR (Proteins with internal repeats) family, which have been characterized previously as covalently bound proteins in *S. cerevisiae* cell wall (Li and Karboune, 2018). In this study, PIR family proteins have not only been detected in *S. cerevisiae* (EB83) but also in *A. pullulans* (FH1). PIR5 (A0A074XJ70) has been described above, as well as the PIR1 protein precursor (C5M4X4) in *C. tropicalis* (AK11). In fact, the Mp65 cell surface mannoprotein was the only putative mannoprotein detected in this study that was not identified in the *S. cerevisiae* EB83 proteome but instead detected in two strains from the *Candida* genus (ECF42, AK11). Although PIR genes were previously thought to be uniquely present in *S. cerevisiae*, an homologous gene has been identified in some yeasts, such as *Yarrowia lipolytica* (Jaafar *et al*., 2003) or *Candida albicans* (Kandasamy *et al*., 2000), and this analysis shows the presence of a PIR-encoded protein in *A. pullulans* and *C. tropicalis* also.

Another group of cell wall proteins identified was the GPI proteins. One of the functions of these proteins is to link other mannoproteins to the β-glucans and also it has been documented its role in biofilm formation and adhesion to other cells or abiotic surfaces (Pittet and Conzelmann, 2007), which has been described in *S. cerevisiae* and *Y. lipolytica*, among others (Lesage and Bussey, 2006; Phienluphon *et al*., 2019). The adhesion capability of these proteins has been highlighted as a pathogenicity factor and also has potential industrial applications, such as the detoxification of certain mycotoxins (Meca *et al*., 2010) or to attach specific components to the cell surface (Phienluphon *et al*., 2019). Finally, it is also interesting that GPI enzymes, which are involved in GPI-anchored protein biosynthesis, were detected (Table 2). It is known that Utr2 and CWH43 could be involved in cell wall remodelling of GPI-anchored proteins (Lesage and Bussey, 2006; Umemura *et al*., 2007).

Proteins related to cell wall polysaccharides biosynthesis

Proteins involved in cell wall carbohydrate (chitin, β-glucans and mannans) metabolism were also characterized. A total of 133 unique proteins were identified among all the strains studied, from which 30 belonged to chitin biosynthesis process (Table 3), 52 to β-glucans (Table 4) and 51 to mannans (Table 5).

Analysis of proteins related to chitin synthesis has revealed the presence of different chitin synthases, chitinases or enzymes that produce chitin precursors in *Candida* sp. (ECF42; AK11), *W. sorbophila* (ECF12) and *S. cerevisiae* (EB83), as well as other proteins which have been associated to chitin biosynthesis process or with its regulation (Table 3). A phosphoacetylglucosamine mutase protein has been identified in six strains (G8BF87; C5MC59; P38628; A0A2T0FNR4; KAA8902178.1). The presence chitinase 2 in AK11 (*C. tropicalis*) and ECF42 (*C. parapsilosis*) and chitin synthase 3 in ECF42 and AK11 could suggest that similar mechanisms are used by the two strains in chitin biosynthesis process.

It is known that the three *S. cerevisiae* chitin synthases are encoded by CHS1, CHS2 and CHS3 genes and *C. albicans* possess four chitinase genes (CHT1, CHT2, CHT3 and CHT4) that can express four different chitin synthases. Nevertheless, these proteins were not detected in *S. cerevisiae* and *Candida* sp. in this work, which could indicate that the expression of these enzymes depends on internal and external cell factors. Moreover, it has been reported that production of these proteins in *S. cerevisiae* and *C. albicans* is affected by the cell growth phase, the pH value and the carbon source, among other factors (Lesage and Bussey, 2006; Yang and Zhang, 2019).

In contrast to the above, only one protein involved in chitin biogenesis from each of FH1 and EB39 strains was detected (Glycosyltransferase family 2 protein - A0A074YGJ8; Glutamine-fructose-6-phosphate aminotransferase - A0A109FLS1 respectively), so this could indicate that these proteins have not yet been annotated within the databases or that their expression is modulated by external factors as was demonstrated in a *Rhodotorula* sp. (Ilyas *et al*., 2016).

In the case of β-glucan biosynthesis, most of the proteins detected were uniquely identified in the individual strains analysed, although some glucan 1,3-β-glucosidases, chaperone DnaK proteins, 1,3-β-glucan synthase component FKS11 and probable family 17 glucosidase SCW4 were shared by different strains as shown in

Table 3. Proteins identified which are involved in chitin biosynthesis and organization.

Protein ID	Strain	Protein name
A0A074YGJ8; KAA8902210.1	*A. pullulans* (FH1); *D. rugosa* (FR19)	Glycosyltransferase family 2 protein
G8BBW5; C5MBZ3	*C. parapsilosis* (ECF42); *C. tropicalis* (AK11)	Chitin synthase 3
G8BHR4; C5MHA2		Chitinase 2
G8BF87; C5MC59; P38628; A0A2T0FNR4; KAA8902178.1	ECF42; AK11; *S. cerevisiae* (EB83); *W. sorbophila* (ECF12); FR19; *D. rugosa* (ECF61)	Phosphoacetylglucosamine mutase
G8BDV8; A0A2T0FCK0	ECF42; ECF12	Chitin biosynthesis protein CHS5
A0A367YFE5	AK11	Glucosamine-fructose-6-phosphate aminotransferase [isomerizing]
C5MIQ6	AK11	Chitin synthase 2
C5M2H4		Cell-wall-associated glycosidase, putative
C5M9H8		Extracellular glycosidase UTR2
KAA8902141.1	FR19	Chitin synthase DEHA2A08822p
KAA8898401.1	FR19; ECF61	Chitin synthase DEHA2C16214p
KAA8903926.1	FR19; ECF61	Glucoseamine-6-phosphate synthase
KAA8904354.1	ECF61	Cell wall protein in family of putative glycosidases Probable xyloglucan endotransglucosylase/hydrolase (fragment)
A0A2T0FJQ3	ECF12	Chitin synthase ChsE
A0A2T0FC74		Chitin synthase-domain-containing protein
A0A2T0FL18		Cts1p
A0A2T0FC51		Chitin synthase export chaperone
A0A2T0FIN2; A0A109FLS1	ECF12; *Rh. mucilaginosa* (EB39)	Glutamine-fructose-6-phosphate aminotransferase
P29311	EB83	Protein BMH1
P53301		Probable glycosidase CRH1
Q05029		Protein BCH1

Table 4. The presence of both 1,3-β-glucan synthases (G8B820; C5MF17; A0A367Y4W5; P38631; Q04952; A0A2T0FG79; KAA8900800.1; KAA8901140.1; C5M9W7; C5M818; P40989) and endo/exo β-1,3-glucanases (A0A074X7Y8; G8B5S1; G8B9Y2; C5MET6), in seven of the eight strains is owing to their essential role in cell wall maintenance and remodelling (Utsugi *et al*., 2002). It is interesting to note that three 1,3-β-glucanosyltransferases from GAS family (GAS1, GAS3 and GAS5) were identified not only in EB83 strain (*S. cerevisiae*) but also in ECF12 (*W. sorbophila*). Enzymatic activity has so far only been proven in GAS1 and GAS5 proteins which are essentially produced during the vegetative phase, while GAS3 is only lowly expressed in that step (Ragni *et al*., 2007). In contrast, a glycosyltransferase family 48 protein (A0A120E8I6) involved in β-glucan metabolism, detected in EB39 (*Rh. mucilaginosa*) strain, shared 57% of its sequence with 1,3-β-glucan synthase component FKS1 found in the model yeast *S. cerevisiae* (blastp analysis: 57% identity, 72% similarity, *E* value 0.0 (https://blast.ncbi.nlm.nih.gov/Blast.cgi)). Furthermore, the Rho GTPase Rho1 was detected in EB39 (A0A109FCX9), as well as in FR19 and ECF61 (KAA8900961.1), and this protein is known to be responsible for regulation 1,3-β-glucan synthase (Kim *et al*., 2016).

In relation to proteins associated with mannan biosynthesis, mannosyltransferases were the most identified ones. Mannose-1-phosphate guanyltransferases (A0A074YKD9; G8BB15; A0A2T0FF15; A0A367YFS0; A0A367YFS0; A0A109FMJ5) were detected in all the non-*Saccharomyces* strains. This protein is required for glycosylation during cell wall synthesis (Agaphonov *et al*., 2001), though other studies indicate that this enzyme catalysed the same reaction in *S. cerevisiae* only in the log phase (Ghaemmaghami *et al*., 2003). Additionally, other enzymes like mannose-6-phosphate isomerase, mannosyl-oligosaccharide 1,2-alpha-mannosidase, Dolichyl-phosphate-mannose-protein mannosyltransferase and mannan polymerase complex subunit MNN9 were identified in three strains or more which are required in critical mannosyl-transfer reactions and in protein glycosylation of mannoproteins (Ghaemmaghami *et al*., 2003).

This analysis has permitted not only the characterization of proteins involved in cell wall polysaccharides biosynthesis of all the strains tested but also could provide a reference for the information collected about polysaccharide concentration described in the next section.

Other proteins involved in cell wall organization, biosynthesis or regulation

Other proteins that are involved in the cell wall organization, biosynthesis or regulation were also detected by proteomics analysis (Fig. 1). The majority of them were

Table 4. Proteins identified which are involved in β-glucans biosynthesis and organization.

Protein ID	Strain	Protein name
A0A074X7Y8	*A. pullulans* (FH1)	Putative Exo-β-1,3-glucanase
A0A074XU23		GTPase SAR1 small G protein
A0A074X5U1		Glycoside hydrolase family 72 protein
A0A074XG47; A0A120E8I6	FH1*; Rh. mucilaginosa* (EB39)	1,3-beta-glucan synthase-Glycosyltransferase family 48 protein
G8B820; C5MF17; A0A367Y4W5; P38631; Q04952	*C. parapsilosis* (ECF42); *C. tropicalis* (AK11); *S. cerevisiae* (EB83)	1,3-β-glucan synthase component FKS1
G8B7X9; G8B842; G8BI93; G8BL41; C5M280; C5MC90; C5M280; C5ME42; P15703		Glucan 1,3-β-glucosidase
G8B5S1	ECF42	Eng1 Endo-1,3-β-glucanase
G8B9Y2		α 1,3-glucosidase
G8BEU5; C5M618	ECF42; AK11	Chaperone DnaK
G8BFV6	ECF42	UTP-glucose-1-phosphate uridylyltransferase
A0A2T0FCJ1	*W. sorbophila* (ECF12)	Glucanosyltransferase-domain-containing protein
A0A2T0FFC4; P22146	ECF12; EB83	1,3-β-glucanosyltransferase GAS1
A0A2T0FDX3; KAA8901546.1	ECF12; *D. rugosa* (FR19; ECF61)	Glycoside hydrolase family 5 protein
A0A2T0FG79	ECF12	Glucan synthesis regulatory protein
A0A2T0FIR7		Probable glucose-regulated protein 78 of hsp70 family
C5M9W7; C5M818	AK11	β-glucan synthesis-associated protein KRE6
C5M972		Septation protein SUN4 precursor
C5MB55		Glucoamylase 1
C5MHA4		Putative glucan endo-1-3-β-D-glucosidase
C5MET6		Endo-1,3(4)-β-glucanase 1
KAA8900800.1	FR19; ECF61	Protein involved in (1,3)-beta-glucan synthesis
KAA8907078.1; KAA8899463.1		1,3-beta-glucanosyltransferase
KAA8900961.1		GTP-binding protein RHO1
KAA8901140.1		Beta-1,3-glucan synthase
KAA8896962.1	FR19	Abg1p
A0A109FCX9	EB39	Rho GTPase Rho1
P52911	EB83	Glucan 1,3-β-glucosidase 2
Q04951		Probable family 17 glucosidase SCW10
Q03655		Probable 1,3-β-glucanosyltransferase GAS3
P53334; KAA8897267.1	EB83; FR19; ECF61	Probable family 17 glucosidase SCW4
P40989	EB83	1,3-β-glucan synthase component GSC2
P23776		Glucan 1,3-β-glucosidase I/II
Q08193		1,3-β-glucanosyltransferase GAS5
P16474		78 kDa glucose-regulated protein homologue

characterized in ECF42 (*C. parapsilosis*), AK11 (*C. tropicalis*) and EB83 (*S. cerevisiae*). The principal group of proteins were kinases which were identified in all the strains tested. These proteins are linked to cell wall integrity, cell division and cell energy homeostasis among other secondary functions (Rubenstein and Schmidt, 2007). Actin cytoskeleton and myosin were also characterized as proteins that belong to cell wall organization although their principal role is related to cell division and cell assembly. On the other hand, different strains had enzymes such as catalases, dehydrogenases and reductases implicated in the stress response. Additionally, enolases and elongation factors, which are involved in the protein biosynthesis, were identified. A small number of proteins were classified as heat shock and pH-responsive proteins and these were only found in two strains. Insenser *et al*. (2010) characterized different proteins from the cell surface of *S. cerevisiae* by MALDI-TOF MS which were also found in this study both in *S. cerevisiae* and non-*Saccharomyces* strains such as in the case of heat shock proteins Hsc82p, which was identified in EB39 strain (A0A109FKA3), and Kar2p, which was present in EB83 strain (P16474). However, the kinases identified were mainly related with glucose and nucleotide metabolism in contrast to the ones characterized in this study that are related with cell wall regulation or biogenesis.

Quantification of cell wall β-glucans and D-mannose

Cell wall polysaccharides were quantified by spectrophotometric measurements. The contents of β-glucans and

Table 5. Proteins identified which are involved in mannan biosynthesis and organization.

Protein ID	Strain	Protein name
A0A074YKD9; G8BB15; A0A2T0FF15; A0A367YFS0; A0A367YFS0; KAA8904063.1; A0A109FMJ5	*A. pullulans* (H1); *C. parapsilosis* (ECF42); *W. sorbophila* (ECF12); *C. tropicalis* (AK11); *D. rugosa* (FR19; ECF61); *Rh. mucilaginosa* (EB39)	Mannose-1-phosphate guanyltransferase
A0A074XPL9; G8BB15; A0A2T0FCF6; C5M519; P29952	H1; ECF42; ECF12; AK11; *S. cerevisiae* (EB83)	Mannose-6-phosphate isomerase
G8B670; KAA8907139.1	ECF42; FR19; ECF61	Mannosyl-oligosaccharide 1,2-alpha-mannosidase
G8B7L9; G8BB96; G8BKX1; A0A2T0FJW1; A0A2T0FJ07; C5M6L9; C5MHX5; C5MFZ7; C5MCP6; M3JZ10; P31382	ECF42; ECF12; AK11; FR19; ECF61; EB83	Dolichyl-phosphate-mannose-protein mannosyltransferase
G8BGU3; G8BIU1; KAA8898421.1; P27809	ECF42; FR19; EB83	Glycolipid 2-alpha-mannosyltransferase
G8BHI9	ECF42	Mannosyl-oligosaccharide glucosidase
G8BGU4	ECF42	α 1,2-mannosyltransferase
A0A2T0FHX7	ECF12	Pmt2p
C5M9U9; A0A2T0FN53; P39107	AK11; ECF12; EB83	Mannan polymerase complex subunit MNN9
A0A2T0FMW5	ECF12	Mannosyltransferase
C5M9U2	AK11	α 1,6-mannosyltransferase
C5MHT8; C5MHT6		β-mannosyltransferase 1
C5M5J2; C5M5U7; KAA8906737.1	AK11; FR19	Mannan endo-1,6-alpha-mannosidase
KAA8908400.1	FR19; ECF61	Mannosyl-oligosaccharide glucosidase (Processing A-glucosidase I) (Glucosidase I)
KAA8907046.1	FR19; ECF61	CIC11C00000001645
A0A125PHL6; A0A120E8X2	EB39	Glycosyltransferase family 39 protein
A0A109FI08		Phosphomannose isomerase type I
A0A109FLS1		Glucosamine-fructose-6-phosphate aminotransferase
P14742	EB83	Glutamine-fructose-6-phosphate transaminase
P32629		Mannan polymerase II complex ANP1 subunit
P46985		Probable alpha-1,6-mannosyltransferase MNN11

D-mannose are represented in Fig. 2. The presence of D-mannose ranged between 8 and 20 g 100 g^{-1} and for β-glucans between 7 and 40 g 100 g^{-1}.

Saccharomyces cerevisiae (EB83) was the strain with the highest concentration of β-glucans (40.1 ± 0.011 g 100 g^{-1}), which was double the value observed in the other strains such as FH1 (*A. pullulans*) and ECF42 (*C. parapsilosis*). In contrast, a similar polysaccharide profile was found between the two *D. rugosa* strains and this could indicate that this may not be a strain-dependent characteristic but is probably associated with species (Van Bogaert *et al*., 2009). Only EB39 (*Rh. mucilaginosa*) had a higher content in mannans (17.9 ± 0.002 g 100 g^{-1}) than in β-glucans (7.3 ± 0.001 g 100 g^{-1}). Proteomic analysis revealed the presence of two proteins involved in β-glucan synthesis in the EB39 strain (A0A120E8I6, A0A109FCX9; Table 4), with more proteins characterized in mannan biosynthesis from this strain (Table 5), supporting the observations in the polysaccharide profile.

In general, the β-glucans are the major cell wall carbohydrate and are mainly composed of 1,3-β-glucans. There were several proteins detected that biosynthesise, catabolize, or modify 1,3 glucans, which is indicative of their prevalence in the cell walls of the strains tested (Table 4). Pengkumsri *et al*. (2016) found that in *S. cerevisiae* this polymer was between 44.5 and 49.2 (% wt./wt.) of the cell wall. Nevertheless, Varelas *et al*. (2016) observed a content up to 64.6%. Similar observations were made by other authors in the case of mannans (Nguyen *et al*., 2018).

The results of previous studies have shown that a mycotoxin binding ability of glucans exists, especially against non-polar molecules such as zearalenone and ochratoxin A, and those slightly hydrophilic like AFB_1, with less affinity compared to the other mycotoxins (Jouany *et al*., 2005). It is known that the helical structure of 1,3-β-glucans is able to trap AFB_1 and higher concentrations are eliminated with longer exposure times (Yiannikouris *et al*., 2006). However, the mycotoxin adsorption by the pure extracts of β-glucans is less

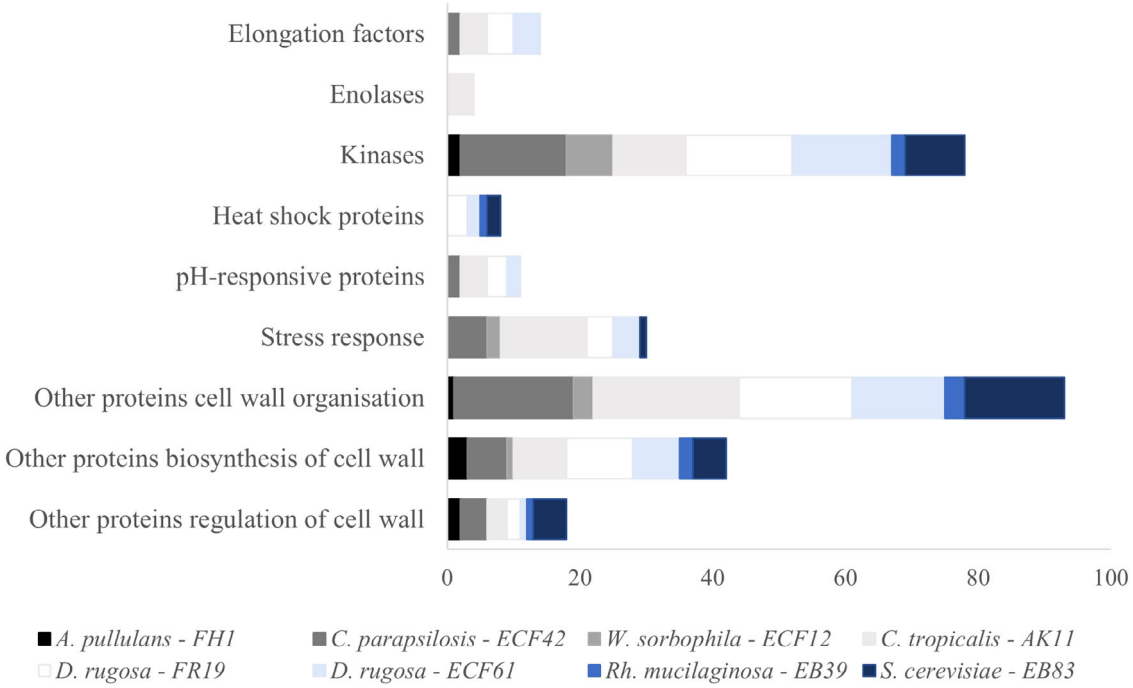

Fig. 1. Other proteins quantified with a role in the cell wall organization, biosynthesis or regulation.

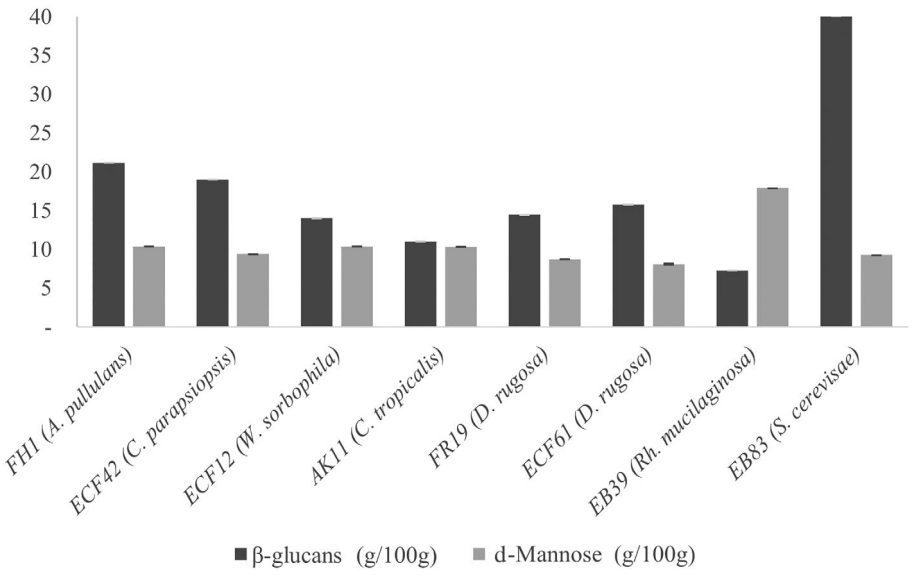

Fig. 2. β-glucans (g/100 g) and D-mannose (g/100 g) cell wall composition in each assayed strain.

efficient than when they are linked to other compounds such as proteins or lipids (Bzducha-Wróbel *et al*., 2019). In the case of mannans, other authors have previously determined that mannans have a positive effect against *Fusarium* mycotoxins when supplied to feeds (Li *et al*., 2012), although one of the principal roles of mannans is the maintenance of the cell wall porosity (Hernawan and Fleet, 1995), which could avoid the introduction of AFB_1 to the intracellular space and its potential toxic effect to the cell.

These results, together with the information obtained in proteomic analyses, have enabled the characterization of cell wall polysaccharides, which are normally involved in mycotoxin elimination. Together with the results obtained in the next section this analysis has permitted to elucidate that the polysaccharides have an important role in

cell wall integrity which could be a key role in avoiding AFB_1 toxicity, as well as, the diverse concentration of them would allow to have different mycotoxin detoxification ability.

Caspofungin sensitivity and cell wall role in AFB$_1$ toxicity

MICs of Caspofungin were quantified for the eight strains studied and different sensitivities were found. Only AK11 (*C*. *tropicalis*) was inhibited by the lowest concentration (0.1 μg ml^{-1}) of Caspofungin followed by EB83 (*S*. *cerevisiae*) with 0.25 μg mlL^{-1}. Additionally, the susceptibility of ECF42 (*C*. *parapsilosis*), ECF12 (*W*. *sorbophila*) and the two *D*. *rugosa* strains (FR19 and ECF61) turned to be the same (0.5 μg ml^{-1}), in contrast to FH1 (*A*. *pullulans*) and EB39 (*Rh*. *mucilaginosa*) which were not inhibited by any of the concentrations tested (0.1, 0.25, 0.5, 0.75, 1 and 2 μg ml^{-1}).

Caspofungin as a fungicidal agent that targets the enzyme complex β-1,3-D-glucan synthase and is effective against *Candida* sp. although its MIC values are species or strain-dependent (Wanjare *et al*., 2016). In relation to *S*. *cerevisiae*, other authors observed the same MIC value reported here (Pérez-Cantero *et al*., 2019). The sensitivity of these strains does not appear to be related to the β-glucan levels in the cell wall, since one of the strains with the highest resistance (FH1) presented the second highest β-glucan levels (Fig. 2). Instead, the protein target of Caspofungin was considered. The cell wall proteome from *S*. *cerevisiae* (EB83), *W*. *sorbophila* (ECF12) and *Candida* sp. strains (AK11 and ECF42) was constituted by several proteins involved in β-glucan biosynthesis, including the Caspofungin target β-1,3-D-glucan synthase FKS1, thus accounting for the sensitivity of these strains to this antimycotic (Letscher-Bru and Herbrech, 2003). In contrast, the resistance presented by *A*. *pullulans* (FH1) and *Rh*. *mucilaginosa* (EB39) might be found in the sequence differences between glycosyltransferase family 48 protein (A0A074XG47 and A0A120E8I6) and their orthologue in *S*. *cerevisiae* (FKS1; P38631) which lead to reduced interaction with echinocandins. Resistance to Caspofungin observed in other yeast species has been related most commonly to amino acid substitution in the hot spots regions of FKS1 or by the presence of multiple FKS isoforms such as in the case of *Fusarium solani* (Ha *et al*., 2006; Lee *et al*., 2018), which could support the hypothesis presented above.

In addition, a toxicity test was carried out in order to determine whether AFB_1 had a toxic effect in cells with a

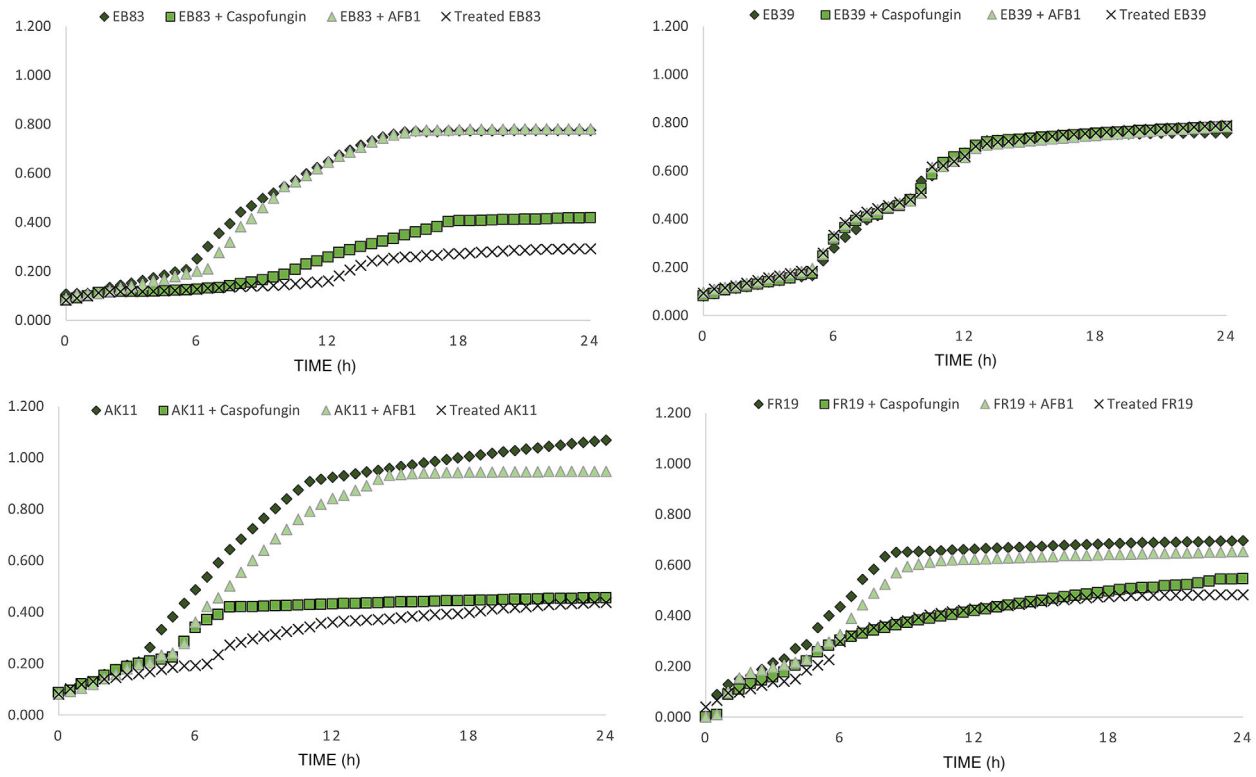

Fig. 3. AFB_1 toxicity kinetics results of EB83 (*S*. *cerevisiae*), EB39 (*Rh*. *mucilaginosa*), AK11 (*C*. *tropicalis*) and FR19 (*D*. *rugosa*) under the conditions tested (Negative control, Caspofungin negative control, AFB_1 negative control and treated sample).

damaged cell wall. Growth curves (OD) for some of the strains examined (EB83, EB39, AK11 and FR19) are shown in Fig. 3.

EB83 (*S. cerevisiae*) and AK11 (*C. tropicalis*) were the strains that showed the highest sensitivity to Caspofungin. Its presence reduced their exponential phase and extended the stationary phase. Growth curves for these strains (in presence of Caspofungin at the MIC plus AFB_1) revealed that there was a toxic effect when the cell wall was damaged, although this toxic effect was higher in the EB83 strain than in AK11 possibly due to its elevated β-glucan content.

A similar growth curve was observed between the FR19 (*D. rugosa*) strain only in the presence of Caspofungin or treated both with Caspofungin and AFB_1. Similar results were found for the other *D. rugosa* (ECF61) (data not shown). It might indicate that, although caspofungin has an effect on the cell wall, AFB_1 would not affect the cell development owing to its absorption by other components from the cell wall. In contrast, EB39 (*Rh. mucilaginosa*) curves proved to be similar despite the presence or absence of Caspofungin. This was due to a resistance to this antimycotic compound, but it also demonstrated that cell wall integrity is essential in toxicity resistance and that β-glucans are not the only cell wall components involved in this process.

Integrity of the yeast cell wall is not only essential for cell protection but also for eliminating mycotoxins as has been reported before (Guo *et al*., 2012; García-Béjar *et al*., 2020a). In certain strains (EB83 and AK11), β-glucan fraction entailed a great percentage of the cell wall composition, which have already been related with AFB_1 adsorption (Bzducha-Wróbel *et al*., 2019). Thus, the absence of these polysaccharides may permit the access of the AFB_1 to the inside of the cell that will produce the toxicity effect observed. Nevertheless, the amount of β-glucans did not seem to be the only explanation for mycotoxin adsorption because is a complex mechanism, which can require other cell wall component such as mannoproteins (Joannis-Cassan *et al*., 2011; Guo *et al*., 2012). Other authors have suggested that mannoproteins would play an important role in aflatoxin binding by *S. cerevisiae* (Joannis-Cassan *et al*., 2011) that could explain the results observed in FR19 and ECF61 (*D. rugosa*) growth curves in presence of AFB_1 and Caspofungin. However, further studies need to be carried out in order to get more information about the specific role of cell wall components (proteins, mannans, chitin…etc.) in AFB_1 detoxification.

Conclusions

This study has provided a more in-depth characterization of the cell wall composition of certain environmental yeast strains with AFB_1 detoxification capability. Structural components such as mannoproteins (PIR family proteins and GPI-anchored proteins) have been identified, which could be potentially involved in mycotoxin elimination. Also, enzymes related to the biosynthesis of polysaccharides have been detected in all the strains tested, although the number of these proteins differed across the strains. Additionally, principal polysaccharides of the cell wall have been quantified and revealing distinct β-glucans and mannans profiles in each strain, with β-glucan levels higher than mannans in all strains except *Rh. mucilaginosa* (EB39).

Strains with higher number of enzymes involved in β-glucan synthesis process displayed a higher sensitivity to Caspofungin (EB83, AK11, ECF12 and ECF42). Intact yeast cell wall preserved the cell from the toxicity effect of AFB_1, as has been observed in Caspofungin-resistant strains (FH1 and EB39).

Although this study gives more information about the cell wall proteome, further studies need to be carried out with the aim of improving protein databases, as well as investigating more about the role of proteins and carbohydrates in detoxification processes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Principal Component Analyses (PCA) plot of the untreated samples and those treated with AFB1 (0.04, 1, 2 or 3 mg L^{-1}).

Fig. S2. Heat map showing the hierarchical clustering from label-free quantitative data for the different samples treated with AFB1 (0.04, 1, 2 or 3 mg L^{-1}) and control samples (NC) for the test carried out with *S. cerevisiae*.

Table S1. Functional identification of cell wall proteins obtained by Blas2Go analysis based on the following Gene Ontology (GO) terms: Cell wall organisation, fungal-type cell wall organisation, fungal-type cell wall organisation or biogenesis, Structural constituent of cell wall, Cell wall, Fungal-type cell wall or Yeast-form cell wall.