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Application of yeasts and yeast derivatives for the biological control of toxigenic fungi and their toxic metabolites

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ABSTRACT

Mycotoxins, the metabolites of toxigenic fungi are important contaminants of food and agriculture industry throughout the world. Among the different strategies to minimize the mycotoxins synthesis and decontamination of food; yeasts and their derivatives are used as efficient biological control agents. In this study, cell walls (CW) of six yeast strains; two commercial baking {*Saccharomyces cerevisiae* (Sc 1 and Sc2)}, two low-fermenting (*Candida intermedia* and *Lachancea thermotolerans*), and two non-fermenting (*Cyberlindnera jadinii* and *Candida friedrichii*) were explored to inhibit *A. flavus* growth, aflatoxin (AF) synthesis and remove mycotoxins from contaminated buffers (at pH 3, 5 and 7) and milk. The volatiles of non-fermenting yeasts showed a significant inhibitory effect on of *A. flavus* growth (up to 79%) and aflatoxin synthesis potential. The highest mycotoxins binding activities of Sc strains were noted against ochratoxin A (92%), AFB2 (66%), AFG2 (59%) and AFB1 (31%). The highest and lowest binding activities occurred at pH 7 and pH 3, respectively. On the other hand, all yeast CWs significantly (44%–54%) reduced aflatoxin M1 (AFM1) from contaminated milk. These results show clearly that yeasts (particularly Sc) can be used as efficient biocontrol and decontamination agents in food industry or the management of mycotoxin contamination.

1. Introduction

Pre- and post-harvest contamination of cereals with toxigenic fungi, and accumulation of mycotoxins in food products remains a big challenge for agriculture and food industry throughout the world (Eskola et al., 2020; Milani, 2013). The synthesis of mycotoxins by fungi depends on several intrinsic and extrinsic factors including environmental humidity, ambient temperature, water activity, pH and nature of the substrate, and most importantly the toxigenic potential of the fungal strain itself (Tola and Kebede, 2016; Hussain et al., 2008). *Aspergillus*, *Penicillium* and *Fusarium* are known as major mycotoxins-producer as the important mycotoxins such as aflatoxins (AFs), ochratoxins (OT), zearalenone (ZEN), fumonisins (FB1, FB2), deoxynivalenol (DON) are synthesized by the fungal strains belonging to these genera (Bhat et al., 2010).

Ingestion of aflatoxin B1 (AFB1) by ruminants results in its biotransformation to aflatoxin M1 (AFM1) which is released in the milk. AFM1 (like its parent AFB1) is known for its carcinogenic, cytotoxic and mutagenic activities, and

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has been placed together with AFB1 as group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2002). Health effects of mycotoxins led food authorities to establish regulatory limits to protect the exposed human and animal categories. Depending on the country/regulatory authority, type of food and possible exposed population the set permissible limits are different. In broader concept, these limits for aflatoxins (B1, B2, G1, G2) are 0–40 μg/kg for food and 0–1000 μg/kg for feed. Similarly, for OTA, these limits are from 0–50 μg/kg for food and 0–1000 μg/kg for feed (Mazumder and Sasmal, 2001). Milk being major diet for infants and young-aged human population, the permissible limits set by European Union (EU) and Gulf Cooperation Countries (GCC) for AFM1 are much lower as 0.05 μg/kg (European Commission, 2010; GCC Standardization Organization, 2013). However, these limits are 10-fold higher (0.5 μg/kg of milk) as set by US Food and Drug Administration (FDA, 2011).

To cope with the issue of mycotoxins and mycotoxicosis, along with improved agricultural practices, several chemical, physical and biological decontamination strategies are currently being employed. Chemical treatment of mycotoxin-contaminated cereals such as, acid, basis, ammonia and other oxidizing and reducing agents have potential to significantly reduce the toxins contents (Karlovsky et al., 2016; Agriopoulou et al., 2020). Application of chemical fungicides is another approach to control the growth and spread of toxigenic fungi on cereal crops and their products. However, chemical associated health risk, their environmental safety and appearance of fungal strains resistant to these fungicides is an emerging issue. Physical methods such as UV irradiation (Jubeen et al., 2012) and mycotoxin binding agents like clays (bentonites, aluminium–calcium silicates), activated charcoal are also being used with variable success (Varga et al., 2010; Bhatti et al., 2018).

Biological control of mycotoxins by employing lactic acid bacteria, yeast and yeast-based products and non-toxigenic fungal species is considered safer and getting popularity in food industry (Tsitsigiannis et al., 2012; Ul Hassan et al., 2019). On one hand, application of microbial volatiles as well as diffusible (non-volatile) compounds against the growth and mycotoxins synthesis of fungi (Higazy et al., 2021; Alasmar et al., 2020; Zeidan et al., 2019; Ul Hassan et al., 2019), and on other hand the use of biologics (such as components of yeast cell wall) for the removal of mycotoxins from the contaminated food and feed matrices (Piotrowska and Masek, 2015), are replacing the conventional mycotoxins managements protocols. In each case, the nature of target toxigenic fungi or mycotoxin plays an important role in the decontamination potential of applied molecules/approach. Mycotoxins removal capacity of biological matrix also depends on other factors such as pH, physical state, temperature and contact duration (Faucet-Marquis et al., 2014).

This *in vitro* study was designed to investigate the effect of yeast volatiles on *A. flavus* growth and its aflatoxin synthesis potential in co-incubation assay. Additionally, to explore the effect of pH and contact duration, yeast cell walls (YCW) from six different isolates were tested for their AFs and OTA binding potential from buffer solutions and milk samples.

2. Materials and methods

2.1. Chemicals and supplies

Citrate- (pH 3), acetate- (pH 5) and phosphate- (pH 7) buffers were prepared in the laboratory as described by Faucet-Marquis et al. (2014). Aflatoxins (AFB1, AFB2, AFG1, AFG2), ochratoxin A (OTA) standards solutions were purchased from Romer Labs (DE, USA). AFM1 (powder) and standards for calibration curve were obtained from Trilogy lab, USA. HPLC grade chemicals used for extraction and analysis of mycotoxins were obtained from Sigma Aldrich, USA. RIDASCREEN® Aflatoxins total ELISA kits were purchased from R-Biopharm, Darmstadt,-Germany.

2.2. Equipment

Acquity ultra performance liquid chromatography (UPLC) coupled with C18 column and fluorescent detector (FLD) were from WATERS (MA, USA). Microplate reader (Thermo Scientific) was used with R-Biopharm software (Z9996 RIDA®-SOFT Win) for data acquisition and calculation of standards curve.

2.3. Microbial strains

In total six yeast strains were used: 2 low-fermenting [*Candida intermedia* 235 (Y235) and *Lachancea thermotolerans* 751(Y751)], 2 non-fermenting [*Cyberlindnera jadinii* 273 (Y273) and *Candida friedrichii* 778Y (Y778] and two commercially available backing yeast *Saccharomyces cerevisiae* (Sc1 and Sc2). Low- and non-fermenting yeast strains were obtained from the collection of Dipartimento di Agraria, Universita di Sassari, Italy. *Aspergillus flavus* AF82 was isolated from animal feed sample in Qatar and identified on the basis of morphological features which was further confirmed by PCR using specie-specific primers (Hassan et al., 2018). The toxigenic potential of the fungi was assessed by exploring the presence of aflatoxin biosynthesis cluster genes as well as *in vitro* mycotoxin synthesis on solid media (Ul Hassan et al., 2019). Yeast extract peptone dextrose agar (YPDA) media was prepared by mixing yeast extract (10 g), peptone (20 g), dextrose (20 g) and agar (15 g) in 1000 ml of water. Yeast-extract peptone broth was prepared same as YPDA without adding agar.

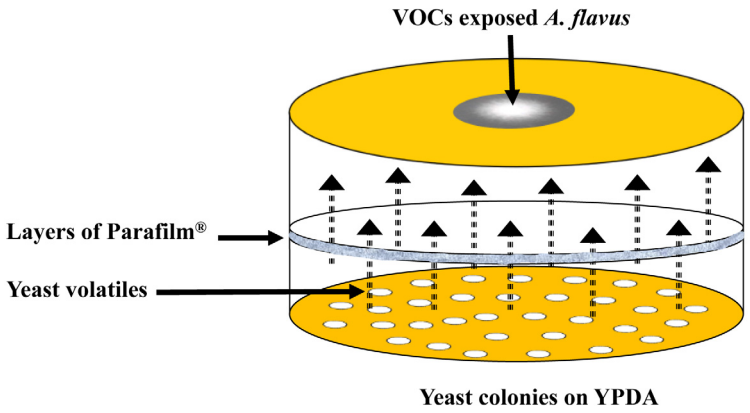

Fig. 1. Pictorial representation of yeasts and *A. flavus* co-incubation experiment. Lower plate having YPDA media was spread with 100 μL of yeast cells suspension. After 24 hrs of incubation, the cover plates were removed and sealed against freshly inoculated fungal spore plate (upper plate). To avoid the leakage of yeast volatiles, sealing was performed with two layers of Parafilm® and an additional layer of Scotch tape. Experiment was continued for 7 days.

2.4. Antifungal activity of yeast volatiles

2.4.1. Effect of yeast VOCs on fungal growth

To explore the effect of yeast volatiles on the growth and mycotoxin synthesis potential of *A. flavus*, co-culture experiments were performed as described by Ul Hassan et al. (2019). Briefly, on YPDA media plates, 100 μl of yeast cells (10^7 cells/ml) were spread and incubated for 24 hrs at 25 °C. At the centre of PDA plate, 10 μL of *A. flavus* spore suspension (10^4 cell/mL) was point inoculated and immediately sealed by replacing the cover plate with growing yeast colonies as represented in Fig. 1. To the control fungi plate, cover plate was replaced with YPDA media plate without inoculated yeast cells. A set of three plates form each treatment was incubated for 3 days at 28 °C for morphological observation. Colony diameter of control (C) and yeast-VOC's exposed fungi (T) were measured in mm, and fungal growth inhibition (%) was calculated as;

$$A.\ flavus\ growth\ inhibition\ (\%) = \frac{(C-T)}{C} X 100$$

2.4.2. Effect of yeast VOCs on aflatoxin synthesis by A. flavus

To determine the effect of exposure to yeasts VOC's on the AFs synthesis potential of *A. flavus*, three fungal inoculated plates were exposed to each test fungi for 7 days as described in Section 2.4.1 above. With the help of sterile cork-borer (7 mm diameter) three colonized media plugs were removed from the yeast-volatiles exposed fungal colonies. To calculate the reduction in mycotoxin synthesis, three plugs were also removed from the control fungal colony (fungi not exposed to yeast VOCs). Collected plugs were weighed and transferred to glass vials for the extraction of AFs in 500 μL of solvent mixture consisting of ethyl acetate, dichloromethane and methanol at 3:2:1, respectively (Hassan et al., 2018). Samples were sonicated for 60 min and 100 μL of the extract was dried using SpeedVac®. Evaporated extract was redissolved in 100 μL methanol (50%) and used in ELISA assay for determination of AFs contents.

2.5. Mycotoxins binding on yeast cell walls

2.5.1. Preparation of yeast cell wall

Yeast cell wall (YCW) were prepared from the yeast cell cultures maintained in YEP media by the method of Bzducha-Wróbel et al. (2014). Briefly, the inoculum (50 μL) from 24 hrs yeast culture was transferred into 200 ml of YEP broth and incubated at 26 °C with constant shaking at 200 rpm. At 48 hrs of incubation the contents of all the flasks were sterilized by autoclaving at 121 °C for 10 min. Yeast-cells pellets were separated by centrifugation at 5000 x *g*, and the supernatant was decanted. To the obtained pellet, 200 ml of distilled water was added. The resuspended pellet was centrifuged again at 5000 x *g*. Each centrifugation step was performed at 4 °C for five min. After removing the supernatant, pellets were washed with increasing concentration of (0.17 mM, 0.34 mM and 85 mM) NaCl to remove the cytoplasmic contents. Each time tubes were centrifuged at 5000 x g for five min. Final washings (2 changes) were performed with distilled water. Obtained residues was completely dried at 80 °C and grind to fine powder to use in the mycotoxins binding experiments.

2.5.2. Mycotoxins binding experiments

AFs, OTA and AFM1 binding potential of the YCW was investigated either in buffer solutions at pH 3, 4 and 7 or milk samples, as described by Faucet-Marquis et al. (2014) with little modifications. Briefly, in Eppendorf tubes 5 mg of YCW were suspended in 990 μl of each buffer for 5 min. Each tube was added with 10 μL solutions of dissolved mycotoxin powder to obtain final concentrations of 0.12, 0.03, 0.12, 0.03, and 0.10 μg/mL for AFB1, AFB2, AFG1, AFG2 and OTA, respectively. For the determination of AFM1 binding, 5 mg of YCWs were added in 990 μL of skimmed milk (tested AFM1-free). After 5 min of incubation, 10 μL of AFM1 suspension was added in the tubes to reach final concentration of 0.10 μg/mL. To the control sets of tubes 5 mg of YCWs were added with 1000 μL buffer solutions or milk samples. For each treatment, three sets of tubes were shake incubated at 37 °C for either 15 min, 30 min or 60 min. After centrifugation at 5000 x g for 5 mins, the supernatants were separated and evaporated using SpeedVac®. The dried residues were solubilized in their respective mobile phase as given below for HPLC analysis. The purpose of exploring the binding of mycotoxins on YCWs at three pH and different incubation duration was to find the suitability of adding yeast for the removal of mycotoxins according to the gastric environment of different animal species.

2.6. Chromatographic conditions

Ultra-high performance liquid chromatography (UPLC) system attached with florescent detector was used for the analysis of mycotoxins. The composition of mobile phase for aflatoxins (other than AFM1) analysis was methanol-acetonitrile-deionized water at 22.5 : 22.5 : 55. Florescent detector was set at 360 excitation and 440 nm emission wavelengths. Similarly, for the analysis of OTA, the mobile phase was consisting of ACN:H2O:CH3COOH (45:54:1) with emission and excitation wave lengths of 460 and 333 nm, respectively. AFM1 analysis was carried out at isocratic mobile phase, prepared by adding water, acetonitrile and methanol at 68 : 24 : 8, respectively. Excitation and emission wavelengths of 360 and 430 nm, respectively were set on FLD detector.

2.7. Statistical analysis

The data related to growth of VOCs exposed fungi was used to calculate growth inhibition (%) as compared to unexposed fungi. All the other data were analysed using analysis of variance test (ANOVA). Obtained means were compared by using *post hoc* Duncan's multiple range test. Means were considered as significant at $p \leq 0.05$. Statistical software (IBM, SPSS, var.23) was used for this purpose.

3. Results and discussion

3.1. Effect of yeast VOCs on A. flavus growth and AFs synthesis

The exposure of *A. flavus* AF82 to yeasts volatiles resulted in significant morphological alteration in terms of colony size (mm) and sporulation (Fig. 2), as measured at day 3 of the experiment. In the control fungi, exposure to YPDA alone had non-significant effect on the colony size (34 ± 1 mm) of *A. flavus* as compared to un-exposed fungi (34 ± 0.7 mm). However, on exposure to volatiles emitted from all six-yeast spp., significant reduction in fungal colony diameter as well as sporulation was observed. The noted inhibition of sporulation in *A. flavus* is in line with the findings of Chang et al. (2015) which was associated with 2-phenylethanol, a major component of yeast volatilome. Colony size of *A. flavus* exposed low-fermenting yeasts Y235 and Y751 was 27 ± 0.9 mm and 28 ± 0.5 mm, respectively as compared to 34 ± 0.7 mm of control fungi. Non-fermenting yeast's (Y778 and Y273) volatiles showed highest antagonistic activity against *A. flavus* growth with colony diameter of 13 ± 0.7 mm and 7 ± 0.2 mm respectively. Baking yeasts Sc1 and Sc2 resulted in *A. flavus* colony diameter of 20 ± 1 mm and 24 ± 0.5 mm, respectively. On exposed to VOCs from Y751, Y235, Y778, Y273, Sc1 and Sc2, the fungal growth inhibition was 20.6%, 17.7%, 61.8%, 79.4%, 41.2% and 29.4%, respectively, as compared to control. In line with the findings of this study, our recent study (Alasmar et al., 2020) with a yeast *Kluyveromyces marxianus* (QKM-4) showed a significant growth inhibition of toxigenic fungi belonging to *Aspergillus*, *Penicillium* and *Fusarium*. The volatile analysis of QKM-4 showed long chain alkanes including well known antifungal molecules nonadecane and eicosane. In the present study, we did not analyse the yeast volatiles for the identification of bioactive molecules responsible for the inhibition of *A. flavus* growth and sporulation. However, in our previous study (Farbo et al., 2018), non-fermenting (Y273 and Y778) and low-fermenting (Y235 and Y751) yeast showed antifungal activity against *A. carbonarius* and GC-MSMS based analysis of head-space VOCs confirmed 2-phenylethanol as antagonistic molecule. *In vivo* experiments by Fiori et al. (2014) with non-fermenting yeast (Y778) against OTA-producing strain of *A. carbonarius* also showed a significant retardation of fungus growth.

In line with the fungal growth inhibition, the co-incubation experiments also showed a significant reduction in the AFs production potential of *A. flavus* (Fig. 3) exposed to non-fermenting yeast volatile (Y778 and Y273). In our previous study, the exposure to these yeast volatiles resulted in significant downregulations in the key genes involved in ochratoxin A synthesis by *A. carbonarius* and *A. niger* and thus decrease in the production of OTA (Farbo et al., 2018). A similar effect on the AFs biosynthesis gene might be involved in *A. flavus* exposed to yeast volatiles. On other hand, in spite of significant inhibitory effects on growth, the fungi exposed to low fermenting yeasts (Y235 and Y751) and two *S. cerevisiae* (Sc1 and

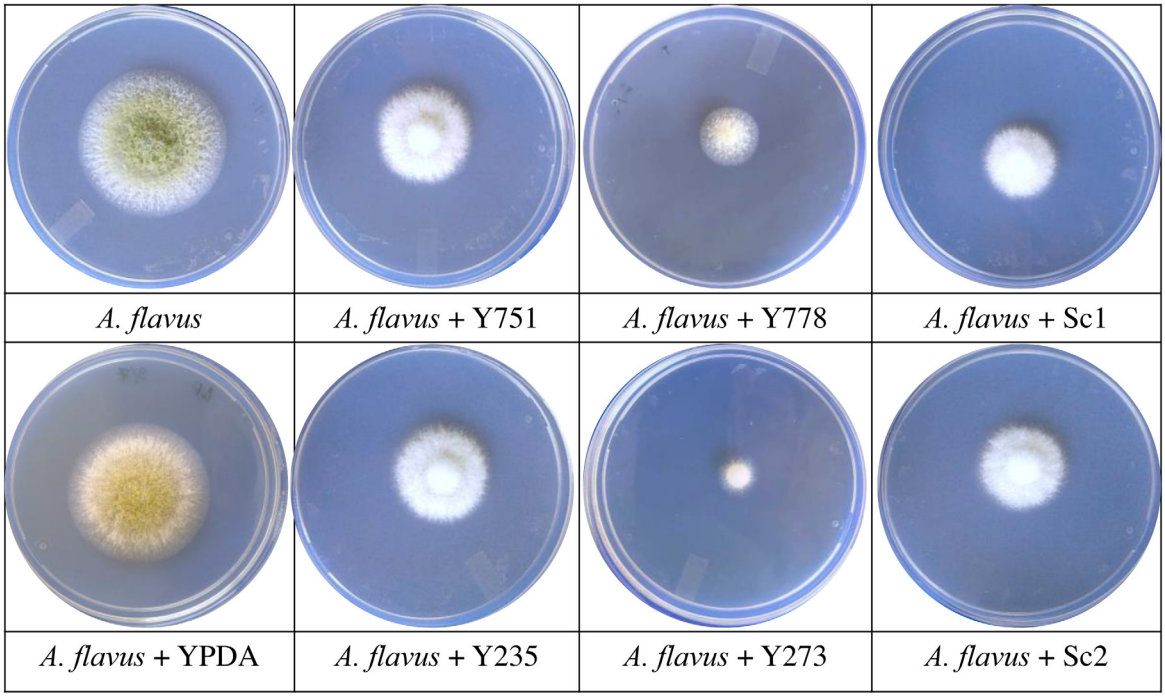

Fig. 2. Colony morphology of *A. flavus* exposed to volatiles emitted from different yeasts. The effect of exposure to VOCs in visible on the colony size and sporulation with maximum antagonistic activity of non-fermenting Y778 and Y273. Representative plates opened at day 3 of exposure to VOCs are presented.

Sc2) showed a non-significant effect on their AFs synthesis potential. Chang et al.(2015) observed that the production of aflatoxin as secondary metabolites results after the cessation of active growth of *A. flavus*, which upregulates the aflatoxigenic genes in fungi. Based on the findings of our previous studies (Farbo et al., 2018; Alasmar et al., 2020), it can be inferred that observed reduction in aflatoxin synthesis by *A. flavus* could be linked with exposure-associated retardation of fungal growth as well as suppression in the pathway genes involved in mycotoxins synthesis.

3.2. Adsorption of mycotoxins on YCW at different pH and incubation durations

3.2.1. Removal of AFB1, AFG1, AFB2 and AFG2

To minimize the health risks associated with dietary exposure to mycotoxins, the use of yeasts and *Lactobacilli*, owing to their 'Generally Considered As Safe (GRAS)' status and wider scavenging abilities is widely accepted (Dawson, 2011; Piotrowska and Masek, 2015; Mil et al., 2015). The efficacy of any mycotoxin absorbent depends on several physical and chemical factors including the nature of mycotoxin, pH of the media, temperature and contact duration, etc. (Devegowda et al., 1998). In the commercial settings, to increase the spectrum of mycotoxin binder, a blend of yeast cells (or products), clays, bacterial enzymes and other substances are supplemented in animal diet. In the present study, the effects of pH of the medium (pH 3, pH 5, pH 7) and incubation duration (15 min, 30 min and 60 min) were investigated on AFs adsorption capacity of YCWs.

After 15 min of co-incubation, a non-significant adsorption of AFB1 by all YCW was observed (Table 1). At pH 7 though not statistically different, a high adsorption of AFB1(14%) by YCW from Sc2 was achieved. The findings of Joannis-Cassan et al. (2011) of relatively lower adsorption of AFB1 on YCW as compared to OTA and ZEA are in line with our results. In contrast to our observations, Kong et al. (2014), reported 92.7% adsorption of AFB1 after 2 hrs of co-incubation in simulated gastric environment of pigs (pH 2.0). These differences might be associated with the nature of yeast, contact duration and/or pH of the medium. In case of AFB2, 15 min of co-incubation resulted in a pH-dependent mycotoxin adsorption by YCW; with maximum (up to 66%) at pH 7 by Sc isolates. In line with these findings, Walters (2012) reported similar adsorption of AFB2 in the intestine of rats co-fed with YCW products. Although there are minor structural differences between AFB1 and AFB2, the adsorption on YCW was much higher for AFB2 than AFB1. The addition of YCW in mycotoxin-contaminated buffer solutions resulted in a non-significant AFG1 adsorption, while AFG2 was adsorbed up to 44% on Sc cell wall at pH 7.

After 30 min of co-incubation, a pH dependent trend in AFB1 adsorption was noted with significantly higher binding on YCW from Sc isolates than others (Table 2). Like AFB1, the adsorption of AFB2 was significantly higher on YCW from Sc2

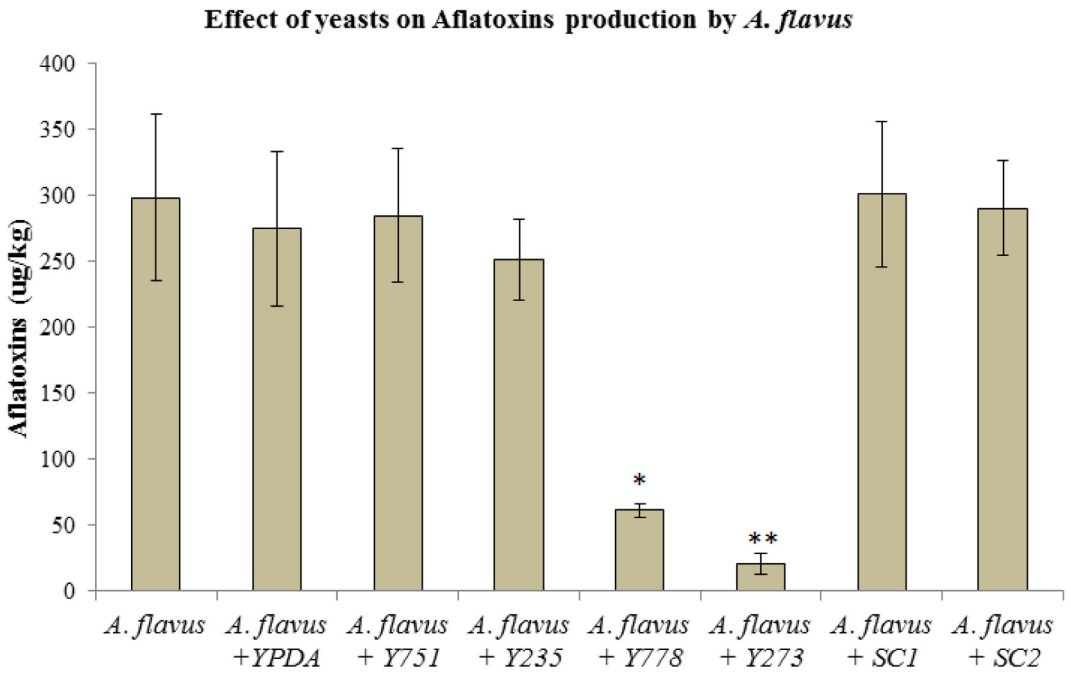

Fig. 3. Effect of yeast volatiles on the aflatoxin production potential of *A. flavus*. In a co-culture experiment a known toxigenic strain of *A. flavus* was exposed to yeast volatiles for 7 days. Colonized media plugs were removed for analysis of AF contents. Each bar represents the mean aflatoxin levels (μg/kg) obtained from five independent samples.

Table 1
Mycotoxin binding potential of yeasts cell walls (YCWs) at different pH (tested after 15 min of co-incubation).

YCW	Citrate buffer (pH 3)				
	AFB1	AFB2	AFG1	AFG2	OTA
Control	0.118 ± 0.010	0.027±0.014^{b}	0.117 ± 0.021	0.027 ± 0.010b	0.100±0.025^{b}
Y235	0.112 ± 0.021 (5)	0.025±0.009^{ab} (7)	0.110 ± 0.026 (6)	0.021±0.008^{ab} (22)	0.041±0.005^{a} (59)
Y751	0.110 ± 0.032 (7)	0.021±0.005^{a} (22)	0.114 ± 0.059 (3)	0.024±0.01^{ab} (11)	0.048±0.008^{ab} (52)
Y273	0.111 ± 0.041 (7)	0.024±0.002^{ab}(11)	0.105 ± 0.034 (11)	0.021±0.008^{ab} (22)	0.046±0.005^{ab} (54)
Y778	0.108 ± 0.026(10)	0.020±0.001^{a} (26)	0.111 ± 0.052 (5)	0.023±0.004^{ab} (15)	0.042±0.010^{a} (58)
Sc1	0.1100.052± (7)	0.021±0.005^{a} (22)	0.103 ± 0.017 (12)	0.020±0.006^{ab} (15)	0.037±0.009^{a} (63)
Sc2	0.112 ± 0.024 (5)	0.024±0.003^{ab}(11)	0.105 ± 0.019 (11)	0.020±0.001^{a} (26)	0.040±0.004^{a} (60)
	Acetate buffer (pH 5)				
Control	0.117 ± 0.081	0.028±0.006^{b}	0.118 ± 0.025	0.027±0.008^{b}	0.100±0.010^{b}
Y235	0.109 ± 0.074 (7)	0.023±0.005^{ab}(18)	0.112 ± 0.039 (5)	0.023±0.006^{ab} (15)	0.039±0.010^{ab} (61)
Y751	0.115 ± 0.036 (2)	0.025±0.010^{ab}(11)	0.120 ± 0.045 (−1)	0.021±0.007^{ab}(22)	0.037±0.008^{ab} (63)
Y273	0.119 ± 0.015 (−1)	0.021±0.006^{a} (25)	0.120 ± 0.007 (−1)	0.025±0.001^{b} (7)	0.035±0.0034^{ab}(65)
Y778	0.119 ± 0.024 (−1)	0.019±0.010^{a} (32)	0.110 ± 0.005 (7)	0.029±0.001^{b} (−1)	0.041±0.004^{ab} (59)
Sc1	0.104 ± 0.095 (11)	0.020±0.001^{ab}(17)	0.108 ± 0.013 (8)	0.021±0.002^{ab} (22)	0.028±0.002^{a} (72)
Sc2	0.106 ± 0.053 (9)	0.021±0.006^{a} (25)	0.107 ± 0.017 (9)	0.019±0.004^{a} (30)	0.029±0.006^{a} (71)
	Phosphate buffer (pH 7)				
Control	0.119 ± 0.041	0.029 ± 0.004c	0.118 ± 0.023	0.027±0.008^{b}	0.100±0.008^{b}
Y235	0.108 ± 0.012 (9)	0.011±0.001^{a} (62)	0.109 ± 0.035 (8)	0.021±0.005^{ab} (22)	0.017±0.004^{ab} (83)
Y751	0.116 ± 0.035 (3)	0.015±0.003^{ab}(48)	0.111 ± 0.062 (6)	0.023±0.006^{ab} (15)	0.013±0.003^{a} (87)
Y273	0.121 ± 0.061 (−1)	0.018±0.004^{b} (38)	0.118 ± 0.052 (0)	0.021±0.005^{ab} (22)	0.012±0.005^{a} (88)
Y778	0.121 ± 0.082 (−1)	0.012±0.006^{a} (59)	0.120 ± 0.041 (−1)	0.024±0.005^{b} (11)	0.018±0.005^{ab} (82)
Sc1	0.108 ± 0.022 (9)	0.010±0.006^{a} (66)	0.108 ± 0.037 (0)	0.018±0.005^{a} (33)	0.008±0.004^{a} (92)
Sc2	0.102 ± 0.012 (14)	0.010±0.007^{a} (66)	0.101 ± 0.082 (14)	0.015±0.001^{a} (44)	0.010±0.002^{a} (90)

Mycotoxin binding on yeast cell wall products at different pH after 15 min of co-incubation. Three buffers at pH 3, 5 and 7 were artificially contaminated with aflatoxins (B1, B2, G1, G2) and OTA and then YCW were added. After co-incubation for 15 mins, mycotoxins contents of the supernatant were determined by UPLC. Values in each column followed by different superscript letters are significantly different from each other. The values in parenthesis indicate the percentage reduction/adsorption of mycotoxin contents.

Table 2
Mycotoxin binding potential of yeasts cell walls (YCWs) at different pH (tested after 30 min of co-incubation).

YCW	Citrate buffer (pH 3)				
	AFB1	AFB2	AFG1	AFG2	OTA
Control	0.118 ± 0.021	0.027±0.005^{b}	0.117 ± 0.052	0.028±0.003^{b}	0.100±0.005^{b}
Y235	0.111 ± 0.025(6)	0.021±0.005^{ab} (22)	0.101 ± 0.056 (14)	0.020±0.004^{ab} (29)	0.043±0.004^{a} (57)
Y751	0.118 ± 0.051 (0)	0.025±0.002^{b} (7)	0.109 ± 0.041 (7)	0.021±0.004^{ab} (25)	0.046±0.003^{ab}(54)
Y273	0.110 ± 0.032 (7)	0.026±0.006^{b} (4)	0.118 ± 0.092 (−1)	0.025±0.003^{b} (11)	0.038±0.005^{a} (62)
Y778	0.116 ± 0.052 (2)	0.019±0.008^{a} (30)	0.120 ± 0.021 (−1)	0.029±0.005^{b} (−1)	0.039±0.004^{a} (61)
Sc1	0.108 ± 0.062 (8)	0.018±0.004^{a} (33)	0.106 ± 0.044 (9)	0.016±0.004^{a} (43)	0.038±0.006^{a} (62)
Sc2	0.104 ± 0.082 (12)	0.019±0.004^{a} (30)	0.105 ± 0.016 (10)	0.018±0.006^{a} (36)	0.040±0.010^{a} (60)
	Acetate buffer (pH 5)				
Control	0.117±0.057^{b}	0.027±0.002^{b}	0.117±0.025^{b}	0.026 ± 0.004	0.100±0.004^{b}
Y235	0.105±0.062^{ab} (10)	0.020±0.004^{ab} (26)	0.112±0.031^{b} (4)	0.024 ± 0.005 (8)	0.040±0.007^{ab} (60)
Y751	0.111±0.035^{b} (5)	0.018±0.006^{ab} (33)	0.110±0.025^{b} (6)	0.025 ± 0.003 (4)	0.038±0.005^{ab} (62)
Y273	0.106±0.025^{b} (9)	0.016±0.006^{a} (40)	0.109±0.056^{b} (7)	0.021 ± 0.001 (19)	0.036±0.006^{ab} (64)
Y778	0.105±0.048^{ab} (10)	0.015±0.003^{a} (44)	0.105±0.066^{ab} (10)	0.023 ± 0.005 (12)	0.040±0.006^{ab} (60)
Sc1	0.101±0.062^{a} (14)	0.018±0.001^{ab} (33)	0.092±0.046^{a} (21)	0.025 ± 0.007 (4)	0.030±0.005^{a} (70)
Sc2	0.095±0.005^{a} (19)	0.014±0.006^{a} (48)	0.080±0.041^{a} (32)	0.021 ± 0.004 (19)	0.032±0.005^{a} (68)
	Phosphate buffer (pH 7)				
Control	0.118±0.055^{b}	0.027±0.006^{b}	0.117 ± 0.028	0.028±0.004^{c}	0.100±0.006^{b}
Y235	0.114±0.063^{b} (3)	0.024±0.004^{b} (11)	0.113 ± 0.032 (3)	0.021 ± 005^{b} (25)	0.016±0.005^{ab} (84)
Y751	0.110±0.075^{b} (7)	0.025±0.006^{b} (7)	0.111 ± 0.051 (5)	0.023±0.003^{bc} (18)	0.018±0.004^{ab}(82)
Y273	0.111±0.081^{b} (6)	0.026±0.003^{b} (4)	0.108 ± 0.056 (8)	0.025±0.006^{c} (11)	0.015±0.003^{a} (85)
Y778	0.116±0.054^{b} (2)	0.021±0.005^{a} (22)	0.105 ± 0.052 (10)	0.021±0.005^{b} (25)	0.018±0.006^{ab} (82)
Sc1	0.085±0.056^{a} (28)	0.021±0.007^{a} (22)	0.106 ± 0.024 (9)	0.018±0.006^{b} (36)	0.010±0.005^{a} (90)
Sc2	0.082±0.041^{a} (31)	0.020±0.004^{a} (26)	0.102 ± 0.034 (13)	0.014±0.007^{a} (50)	0.012±0.001^{a} (88)

Mycotoxin binding on yeast cell wall products at different pH after 30 min of co-incubation. Three buffers at pH 3, 5 and 7 were artificially contaminated with aflatoxins (B1, B2, G1, G2) and OTA and then YCW were added. After co-incubation for 30 mins, mycotoxins contents of the supernatant were determined by UPLC. Values in each column followed by different superscript letters are significantly different from each other. The values in parenthesis indicate the percentage reduction/adsorption of mycotoxin contents.

isolate in all tested pH buffers. Unlike AFG2, the adsorption of AFG1 was higher at pH 5 on YCW from Sc2 than control. Sc isolates showed significantly higher adsorption of AFG1 at all three-pH tested, while Y253, Y273, Y778 at pH 3 and Y273 at pH 7 showed significantly higher adsorption than control. A noted non-significant effect of increasing the incubation duration on the mycotoxin absorption potential of yeast cell wall has been earlier reported by Pizzolitto et al. (2012) and Shetty et al. (2007). In general, the binding process is quick and takes place in 1–30 min (Faucet-Marquis et al., 2014), so increasing the incubation during might not have significant effect on binding potential of yeast.

After 60 min of incubation, AFB1 adsorption was significantly higher at pH 7 by YCW from Sc isolates (Table 3). While both isolates of Sc showed significantly higher adsorption of AFB2 at all three pH points. Unlike pH 3, the adsorption of AFG1 was higher at pH 7 by Sc isolates. AFG2 was adsorbed significantly higher on YCW from Sc isolates at all tested pH. Except Y253 at pH 3 and Y751 at pH 5, at all the yeast isolates at all tested pH showed higher adsorption than in the control group. Comparable data, on the binding of AFG1 and AFG2 by YCW are not found in the accessible published literature. However, from these findings it is evident that minor structural difference in mycotoxins can affect the adsorption efficacy of the binding agent. The nature of interaction of binders with target mycotoxin remains the topics of debates, however the explanation by Jouany et al. (2005) of weak hydrogen and van der Wall bonds between yeast cell wall and interacting mycotoxins is widely accepted. These interactions are more like "adsorption" rather than "binding" and are prone to reversibility depending on changing gastric environment (such as pH) throughout the length of gastro-intestinal tract (Vartiainen et al., 2020).

3.2.2. Removal of OTA by YCWs from contaminated buffer solutions

The binding potential of OTA from contaminated solutions by yeast cells and their cell wall products depends on several factors including pH of the media and thickness of the wall (Armando et al., 2012; Faucet-Marquis et al., 2014). In the present study, among the tested mycotoxins, best adsorption of OTA was noted and showed a significant effect of the pH of the media. At pH 7, highest amount (92%) of OTA was adsorbed by Sc1 at incubation of 15 min (Table 1). Like other mycotoxins, increasing the incubation duration beyond 15 min had non-significant effect on the OTA binding potential of the YCWs (Tables 2 and 3). In contrast to the recent findings of Vartiainen et al. (2020), where highest adsorption of OTA was noted at pH 2.5 and was revered at higher pH of 6.5, in the present study OTA-binding efficacy of YCWs was higher in buffer at pH 7 as compared to pH 3. However, in line with our results, Piotrowska and Masek (2015) observed that highest adsorption of OTA on YCWs is achieved at pH closer to neutral, whereas acidic and basic pH changes the conformation of glucans leading to decrease in the binding potentials of the cell walls.

Table 3
Mycotoxin binding potential of yeasts cell walls (YCWs) at different pH (tested after 60 min of co-incubation).

YCW	Citrate buffer (pH 3)				
	AFB1	AFB2	AFG1	AFG2	OTA
Control	0.117 ± 0.025	0.026±0.005^{b}	0.119 ± 0.062	0.027±0.005^{b}	0.100±0.004^{b}
Y235	0.115 ± 0.036 (2)	0.021±0.006^{ab} (19)	0.111 ± 0.032 (7)	0.025±0.004^{b} (7)	0.040±0.005^{a} (60)
Y751	0.112 ± 0.025 (4)	0.024 ± 005^{b} (8)	0.119 ± 0.024 (0)	0.021±0.005^{ab} (22)	0.036±0.002^{ab} (54)
Y273	0.114 ± 0.092 (3)	0.026±0.005^{b} (0)	0.102 ± 0.025 (14)	0.025±0.007^{b} (7)	0.031±0.010^{a} (59)
Y778	0.111 ± 0.087 (5)	0.020±0.002^{ab} (23)	0.108 ± 0.025 (9)	0.028±0.008^{b} (−1)	0.042±0.003^{a} (58)
Sc1	0.106 ± 0.056 (9)	0.014±0.004^{a}(46)	0.105 ± 0.062(12)	0.018±0.005^{a} (33)	0.039±0.002^{a} (61)
Sc2	0.103 ± 0.054 (12)	0.015±0.005^{a} (32)	0.106 ± 0.042 (11)	0.015±0.001^{a} (44)	0.042±0.005^{a} (58)
	Acetate buffer (pH 5)				
Control	0.118 ± 0.008	0.028±0.005^{c}	0.116 ± 0.007	0.027±0.005^{b}	0.100±0.005^{b}
Y235	0.114 ± 0.085 (3)	0.021±0.004^{b} (25)	0.110 ± 0.021 (5)	0.021±0.004^{ab} (22)	0.041±0.010^{ab} (59)
Y751	0.113 ± 0.076 (4)	0.018±0.006^{ab} (36)	0.108 ± 0.052 (7)	0.025±0.006^{b} (7)	0.038±0.004^{a} (62)
Y273	0.115 ± 0.058 (3)	0.016±0.004^{ab} (43)	0.105 ± 0.035 (9)	0.024±0.006^{b} (11)	0.036±0.006^{a} (64)
Y778	0.116 ± 0.025(2)	0.014±0.005^{ab} (50)	0.104 ± 0.041 (10)	0.020±0.004^{ab} (26)	0.036±0.006^{a} (64)
Sc1	0.105 ± 0.054 (11)	0.012±0.001^{a} (57)	0.108 ± 0.052 (7)	0.014±0.007^{a} (48)	0.030±0.012^{a} (70)
Sc2	0.104 ± 0.065 (12)	0.010±0.003^{a} (64)	0.104 ± 0.028 (10)	0.011±0.005^{a} (59)	0.029±0.005^{a} (71)
	Phosphate buffer (pH 7)				
Control	0.126±0.062^{b}	0.025±0.006^{b}	0.124±0.062^{b}	0.026±0.005^{b}	0.100±0.007^{b}
Y235	0.110±0.035^{ab} (13)	0.020±0.004^{ab} (20)	0.110 ± 0.024ab (11)	0.024±0.005^{b} (8)	0.018±0.005^{a} (82)
Y751	0.109±0.025^{ab} (13)	0.018±0.005^{ab} (28)	0.111±0.062^{ab} (10)	0.021±0.004^{ab} (19)	0.018±0.005^{a} (82)
Y273	0.108±0.062^{ab} (14)	0.015±0.005^{a} (40)	0.108±0.014^{ab} (13)	0.023±0.010^{b} (12)	0.015±0.002^{a} (85)
Y778	0.115±0.036^{b} (9)	0.017±0.002^{a} (32)	0.106±0.053^{ab} (15)	0.024±0.006^{b} (8)	0.012±0.001^{a} (88)
Sc1	0.100±0.045^{a} (21)	0.015±0.008^{a} (40)	0.100±0.012^{a} (19)	0.018±0.008^{a} (31)	0.009±0.008^{a} (91)
Sc2	0.098±0.062^{a} (22)	0.014±0.003^{a} (44)	0.095±0.008^{a} (23)	0.014±0.006^{a} (46)	0.010±0.006^{a} (90)

Mycotoxin binding on yeast cell wall products at different pH after 60 min of co-incubation. Three buffers at pH 3, 5 and 7 were artificially contaminated with aflatoxins (B1, B2, G1, G2) and OTA and then YCW were added. After co-incubation for 60 mins, mycotoxins contents of the supernatant were determined by UPLC. Values in each column followed by different superscript letters are significantly different from each other. The values in parenthesis indicate the percentage reduction/adsorption of mycotoxin contents.

Table 4
Adsorption of AFM1 on yeast cell walls (YCW).

YCW	Incubation time		
	15 min	30 min	45 min
Control	0.100±0.009^{c}	0.100±0.010^{c}	0.100±0.007^{c}
Y235	0.052±0.010^{ab} (48)	0.054±0.007^{ab} (46)	0.052±0.008^{ab} (48)
Y751	0.088±0.011^{b} (12)	0.083±0.012^{b} (17)	0.084±0.010^{b} (16)
Y273	0.046±0.008^{a} (54)	0.044±0.002^{a} (56)	0.045±0.011^{a} (55)
Y778	0.056±0.008^{ab} (44)	0.058±0.009^{ab} (42)	0.057±0.012^{ab} (43)
Sc1	0.048±0.006^{a} (52)	0.047±0.013^{a} (53)	0.046±0.010^{a} (54)
Sc2	0.049±0.007^{a} (51)	0.048±0.008^{a} (52)	0.048±0.010^{a} (52)

Adsorption of AFM1 from artificially contaminated milk by yeast cell walls. Skimmed milk samples were spiked with 0.10 μg/mL and added with 5 mg of YCW. The levels of AFM1 in the supernatants were determined after 15, 30 and 60 min of incubation at 37 °C. Values in each column followed by different superscript letters are significantly different from each other. The values in parenthesis indicate the percentage reduction/adsorption of mycotoxin contents.

3.2.3. Removal of AFM1 by YCWs from contaminated milk

Addition of YCW to AFM1-spiked milk samples, resulted in significant removal (up to 56%) of toxins (Table 4). The highest AFM1 binding activity of cell wall from a non-fermenting Y273 (56%) was followed by Sc strains (51%–54%). As with the other mycotoxins, the effect of increasing the incubation time was non-significant on the adsorption capacity of the YCWs. Removal of AFM1 from contaminated milk and other dairy products with live yeast, yeast cell walls and *Lactobacilli* spp., have been reported by several researchers (Elsanhoty et al., 2014; Serrano-Niño et al., 2013; Corassin et al., 2013; Karazhiyan et al., 2016). Ismail et al. (2017) and (Corassin et al., 2013) recorded a much higher efficacy of Sc cell walls which removed 100% and 92% of AFM1 from milk, respectively. The differences in the binding potential of the biological substances are associated with various physical, chemical, environmental and other factors including the duration of interaction, temperature and pH of the medium.

4. Conclusion

Based on the findings of this study, it can be inferred that yeast volatiles can be used to minimize the vegetative growth and mycotoxin synthesis potential of toxigenic fungi. However, to clearly understand the nature and mode of action, further studies towards the analysis of yeast volatilome and investigation into their *in vivo* interaction for the application on food crops against toxigenic fungi are needed. In addition, the observed mycotoxins removal potential of YCWs from contaminated buffers and milk, highlights their perspective economical and safer application in food and industry. The spectrum of binding potential of YCWs at different pH against AFs, OTA and AFM1 showed their suitability for application in different food/feed matrices and gastric environment of different animal species. Further *in vivo*/simulated gastro-intestinal environment studies with *Fusaria* mycotoxins will provide a comprehensive picture of their suitability for animal species.

CRediT authorship contribution statement

Zahoor Ul Hassan: Conceptualization, Methodology, Validation, Analysis of results, Writing and reviewing. **Roda Al Thani:** Conceptualization, Writing and reviewing. **Fathy A. Atia:** Methodology. **Mohammed Alsafran:** Methodology, Validation, Analysis of results. **Quirico Migheli:** Conceptualization, Validation, Analysis of results, Writing and reviewing. **Samir Jaoua:** Conceptualization, Methodology, Validation, Analysis of results, Resources, Writing and reviewing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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