

Reduction of Ochratoxin A Levels in White Wine by Yeast Treatments

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ABSTRACT

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This paper describes the abilities of 21 yeast strains isolated from six different wine-grapes of Turkey to bind ochratoxin A (OTA). Viable (10^8 CFU/mL) and heat-treated yeast cells were incubated both in phosphate-buffered saline (PBS) and white wine containing 10 ng OTA per mL for 4 h at 25°C. After centrifugation, the concentration of OTA was measured in the supernatant fraction using a high-performance liquid chromatography system coupled with fluorescence detector. The adsorption abilities of OTA by viable yeast strains within 4 h ranged from 1.96 to 26.11% in PBS. On the other hand, a slight decrease was observed in the percentage of OTA removal by yeast strains in white wine when compared to their activity in PBS. The addition of yeasts at 10^8 CFU/mL resulted in a reduction to a maximum of 21.40% in white wine, with respect to the control. Among the yeasts, *Candida famata* D7 was found to be the most efficient binder to OTA both in spiked white wine and PBS. In addition, dead yeast cells can potentially be used for removing OTA (a maximum of 30.45%) from white wine.

Key words: adsorption, high-performance liquid chromatography, ochratoxin A, yeast.

INTRODUCTION

Ochratoxin A (OTA) is a secondary metabolite produced by mainly *Penicillium verrucosum* in temperate climates and *Aspergillus ochraceus* and the rare *Aspergillus carbonarius* in warm and tropical regions⁹. It has been found as a contaminant in cereals, beer, coffee beans, cacao, spices, dried wine fruit, grape juice and wine, as well as in human blood and animal tissues⁵. In the European diet, wine has been identified as the second major source of human exposure to OTA, following cereals, corresponding to 15%¹⁷.

OTA causes carcinogenic, teratogenic, immunotoxic, genotoxic and possibly neurotoxic effects in some laboratory animals⁵. Moreover, it is a well-known nephrotoxic agent and has been associated with fatal human kidney disease, referred to as Balkan Endemic Nephropathy and with an increased incidence of tumours of the upper uri-

nary tract¹⁰. The toxin was evaluated by The Scientific Committee on Food, who proposed that exposure should be below 5 ng/kg b.w./day⁸. In 2001, The Joint FAO/WHO Expert Committee on Food Additives set a provisional tolerable weekly intake (PTWI) of 100 ng/kg b.w., corresponding to approximately 14 ng/kg b.w. per day¹⁰. It was also classified by the International Agency for Research on Cancer (IARC) of WHO as a group 2B (possible human carcinogen) agent in 1993¹², based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans.

Several physical, chemical and biological methods have been tested for the prevention and/or reduction of mycotoxin contamination in several food products. Even though certain treatments have been shown to reduce the levels of specific mycotoxins, it is clear that there is no single currently available method which would destroy all mycotoxins in foods without leaving their metabolites and without changing the nutritional value of food. It should be also noted that chemical treatment is not allowed within the EC for commodities destined for human consumption¹³. This has led to the search for alternative strategies such as biological agents. Many different species and strains of lactic acid bacteria have been tested for the removal of aflatoxins and other mycotoxins from aqueous solution and food model. El-Nezami et al.⁷ showed that *Lactobacillus rhamnosus* GG and *L. rhamnosus* strain LC-705 were able to remove approximately 80% of aflatoxin B₁ (AFB₁). In a similar study, Pierides et al.¹⁶ observed that probiotic bacteria removed up to 50.7% of AFB₁ from phosphate-buffered saline (PBS). In another research conducted by Turbic et al.¹⁹, the removal of OTA from buffered solution by *L. rhamnosus* GG and *L. rhamnosus* LC-705 was found to be 47.12% and 36.43%, respectively within 1 h. Similarly, Kabak and Var¹⁴ found that the binding abilities of AFM₁, by *Lactobacillus* and *Bifidobacterium* strains in PBS and reconstituted milk, ranged from 25.7 to 32.5% and from 21.2 to 29.3%, respectively.

In recent years, yeasts have also been reported to have high adsorption ability against mycotoxins in aqueous solution. Cecchini et al.⁴ demonstrated that the percentage of OTA removal during fermentation was between 46.83% and 52.16% in white wine and between 53.21% and 70.13% in red wine, depending on the yeast strain used. In another study conducted by Shetty and Jespersen¹⁸, most of the yeast strains (10^8 cells) bound more than 15% of AFB₁ within 72 h and the toxin binding was highly strain specific. Similarly, Caridi et al.² have reported that the removal of OTA in wines by 20 different *Saccharo-*

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myces sensu stricto strains, using a naturally and spiked OTA-containing grape must (1.58 and 7.63 ng/mL, respectively), after 90 days of fermentation were between 39.9-92.1% and between 67.9-83.4%, respectively.

Therefore, the primary aim of this study was to determine the removal ability of OTA from PBS and white wine by yeasts isolated from wine-grapes of Turkey. The effect of cell viability on the removal of OTA from PBS and wine was also evaluated.

MATERIALS AND METHODS

Grape samples

During the 2005 season, grapes were harvested from three important wine-producing regions in Turkey, which belong to central Anatolia, east Anatolia and Aegean region. Six grape varieties were included: Emir, Dimrit, Kalecik Karası (central Anatolia), Bogazkere and Okuzgozu (east Anatolia) and Sultaniye (Aegean region). Bunches were collected in sterile bags, transported to the laboratory and stored at 4±1°C until required for analysis.

Isolation and identification of yeasts from grapes

For each grape variety, a sample using 10 g of berries randomly selected was prepared by transferring aseptically into 90-mL sterile peptone water (0.1% w/v) and mixed thoroughly. Each suspension (0.1 mL) was plated over both yeast extract agar (Merck, Germany) and malt extract agar (Merck, Germany) media. All plates were incubated for 3 days at 25°C. The strains were purified and maintained in yeast extract tubes under refrigeration, covered with sterile paraffin to reduce their metabolic activity and to avoid agar dehydration.

The isolates were identified using the API 20°C test strips (Biomérieux, France). The identification by the API 20°C system is based on 19 carbohydrate assimilation tests. The experiments were carried out according to the manufacturer's instructions. Inoculation of the tubes was

performed by adding a yeast suspension with a turbidity equivalent to a 2 McFarland to the dehydrated substrates. The strips were incubated at 30°C in a plastic incubation tray and then read for the presence of growth (turbidity) at 24, 48 and 72 h. The results were transformed into a numerical profile which was compared with those given in the profile list in the APILAB identification package program. The identification of yeast strains isolated from Turkish wine-grapes by API 20°C system is indicated in Table I.

OTA standard

OTA standard was obtained from Sigma-Aldrich (USA) as a crystalline powder form. A stock solution (approximately 500 µg/mL) was prepared by dissolving 1 mg of OTA in 2 mL of toluene/acetic acid (99/1, v/v). The solution was left overnight at room temperature to ensure complete dissolution of the crystalline OTA. A 50 µL of OTA standard stock solution was transferred to a 25 mL brown volumetric flask and was quickly diluted with toluene/acetic acid to obtain a working solution, at 1 µg/mL. Standard working solution was stored at -18°C in the dark; the working solution was freshly prepared and held for less than 1 month.

Chemicals

All solvents were analytical grade and were obtained from Merck (Darmstadt, Germany). PBS was prepared by dissolving 0.2 g KCl, 0.2 g KH₂PO₄, 1.16 g anhydrous Na₂HPO₄, and 8 g NaCl in 900 mL distilled water. The pH of the PBS buffer was adjusted to 3.5 with 0.1 M HCl and diluted to 1000 mL. In all analytical steps, highly purified water generated by a Millipore Synergy 18S Ultra-Pure Water System from Millipore (Guyancourt, France) was used.

OTA removal assay with contaminated PBS

A volume of the culture broth corresponding to 10⁸ yeast/mL was centrifuged (3000xg, 15 min) and the pellets were washed with water. The pellets were suspended in 5 mL PBS containing 10 ng/mL OTA. Tubes were mixed and the suspensions were incubated at 25°C for 4 h. An OTA positive control (10 ng/mL in PBS) in the absence of yeasts and negative controls (yeasts suspended in pure PBS) were also incubated at the same time points to monitor the efficacy of yeasts in the binding OTA. At the end of the incubation period all tubes were centrifuged at 3000 × g (Heraeus, Labofuge 200) for 15 min, then the supernatants were collected and transferred to clean tubes. The tubes were stored at 4±1°C until OTA analysis. The unbound OTA contents of the supernatants were determined by HPLC with fluorescence detection. All experiments were performed in triplicate. To determine the effect of cell viability on the binding affinity to OTA, yeast cells (10⁸ CFU/mL) were heated at 90°C for 15 min. The dead yeasts were pelleted and contaminated with OTA as described above.

OTA removal assay with contaminated wine

White wine-OTA free samples were contaminated with standard working solution of OTA at 10 ng/mL. Both viable (10⁸ CFU/mL) and heat-killed yeasts were treated as

Table I. Yeast strains identified in Turkish wine-grapes during 2005 season and their source.

Yeast	Source
<i>Candida famata</i> E1	Emir
<i>Candida lusitanae</i> E2	Emir
<i>Kloeckera</i> spp. E3	Emir
<i>Kloeckera</i> spp. E4	Emir
<i>Candida famata</i> E6	Emir
<i>Candida famata</i> D1	Dimrit
<i>Candida lusitanae</i> D2	Dimrit
<i>Rhodotorula glutinis</i> D6	Dimrit
<i>Candida famata</i> D7	Dimrit
<i>Candida lusitanae</i> D9	Dimrit
<i>Candida lusitanae</i> D11	Dimrit
<i>Candida lusitanae</i> B1	Boğazkere
<i>Kloeckera</i> spp. B2	Boğazkere
<i>Kloeckera</i> spp. B3	Boğazkere
<i>Cryptococcus laurentii</i> B4	Boğazkere
<i>Candida lusitanae</i> O1	Öküzgözü
<i>Candida famata</i> O3	Öküzgözü
<i>Candida lusitanae</i> KK1	Kalecik Karası
<i>Kloeckera</i> spp. KK2	Kalecik Karası
<i>Candida lusitanae</i> KK4	Kalecik Karası
<i>Candida guilliermondii</i> S1	Sultaniye

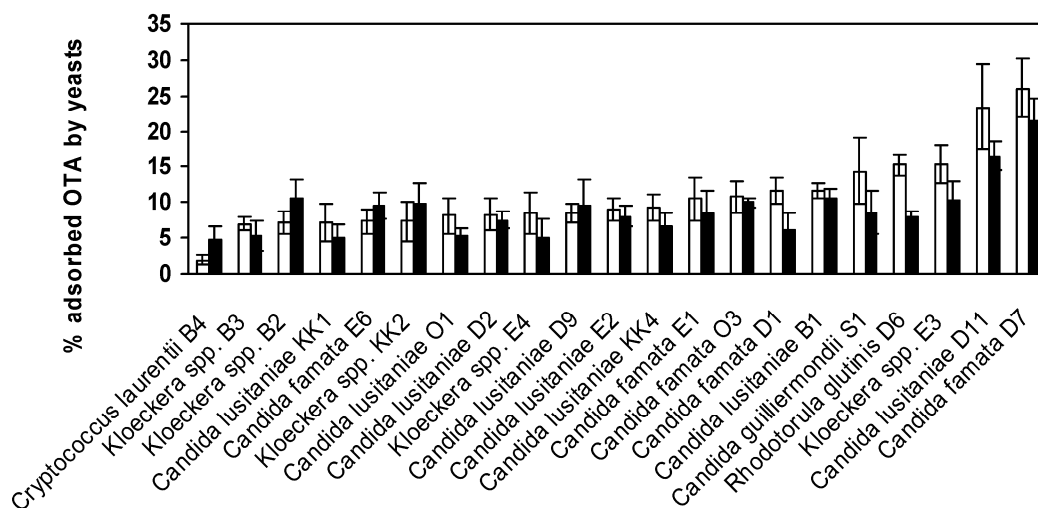


Fig. 1. Adsorption abilities of viable yeasts isolated from Turkish wine-grapes within 4 h (in PBS, □; in white wine, ■).

described earlier, but instead of PBS, the pellets were suspended in spiked white wine. Again all removal assays were carried out in triplicate.

HPLC analysis

The residual OTA content of the supernatants was determined by HPLC (Agilent 1100 HPLC system) consisting of a CSI-6150 online vacuum degasser (Cambridge Scientific Instruments, England), isocratic pump (G 1310 A9, Agilent) and fluorescence detector. Data were recorded on a personal computer equipped with Chemstation software. The separation was conducted isocratically using a Silica 5 µm ACE 5 C18, 100 Å, 25 × 46 mm column supplied by Advanced Chromatography Technologies (Scotland). A Rheodyne 7725i stainless steel manual injector (Agilent, USA) with 20 µL loop was used. Run time for samples was 20 min using a flow rate of 1 mL/min, with a mobile phase of 47:51:2 (v/v/v) acetonitrile/water/acetic acid. All supernatant samples filtered through a 0.45 µm syringe filter (Millipore, USA) prior to injection onto the HPLC column by 50 µL syringe (Hamilton, Switzerland). Two injections were performed for each sample. The excitation and emission wavelengths of the fluorescence detector (Agilent 1100) were set to 333 and 470 nm, respectively. The retention time of OTA was approximately 13 min. A recovery percentage was calculated for PBS and white wine individually. Mean recoveries for PBS and white wine were 87.64% and 84.98%, respectively. The percentage of OTA bound to the yeasts was calculated using the formula:

$$100\% \times (1 - \frac{\text{peak area of OTA}_{\text{in the supernatant}}}{\text{peak area of OTA}_{\text{in the positive control}}})$$

Statistical analysis

The results of the adsorption assays were subjected to one-way analysis of variance (ANOVA) using the SPSS 10.0 statistical package program. Duncan multiple comparisons were used to determine significant differences for OTA binding among strains. A Student's t test was also

used to test significant differences between the results of OTA binding assays for viable and treated yeast cells. Probability (*P*) values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Microbiological survey of six samples of wine-grapes of Turkey showed the presence of four genera (Table I). The yeasts isolated belonged primarily to the genera *Candida* and *Kloeckera*, together with species of *Cryptococcus* and *Rhodotorula*. The species identified within the *Candida* genus were: *C. famata*, *C. lusitanae* and *C. guilliermondii*.

The adsorption abilities of OTA by viable yeast strains isolated from wine-grapes of Turkey are shown in Fig. 1. The adsorption abilities of OTA by viable yeast strains were found to range from 1.96 to 26.11% and from 4.75 to 21.40%, in PBS and white wine, respectively, within 4 h. When compared to other viable yeast strains, viable *C. famata* D7 and *C. lusitanae* D11 showed significantly better (*P* < 0.05) ability to remove OTA from PBS (26.11% and 23.46%, respectively). However, the adsorption of OTA in PBS was lowered to 1.96% in the presence of *Cryptococcus laurentii* B4. A slight decrease was observed in the percentage of OTA removal by viable yeast strains in white wine when compared to their activity in PBS. Among the yeast strains, viable *C. famata* D7 and *C. lusitanae* D11 adsorbed significantly more OTA in white wine (*P* < 0.05). The other 19 strains exhibited lower abilities.

Our study demonstrated that ranging from 4.10 to 31.31% and from 8.08 to 30.45%, OTA was adsorbed by the heat-killed cells in PBS and white wine, respectively, within 4 h (Fig. 2). There were significant differences (*P* < 0.05) among the heat-killed yeast strains for the adsorption ability both in PBS and white wine; strain *C. famata* D7 exhibited the highest capacity to remove OTA, while strain *Cry. laurentii* B4 showed the lowest capacity. In accordance to our study, the differences in the adsorp-

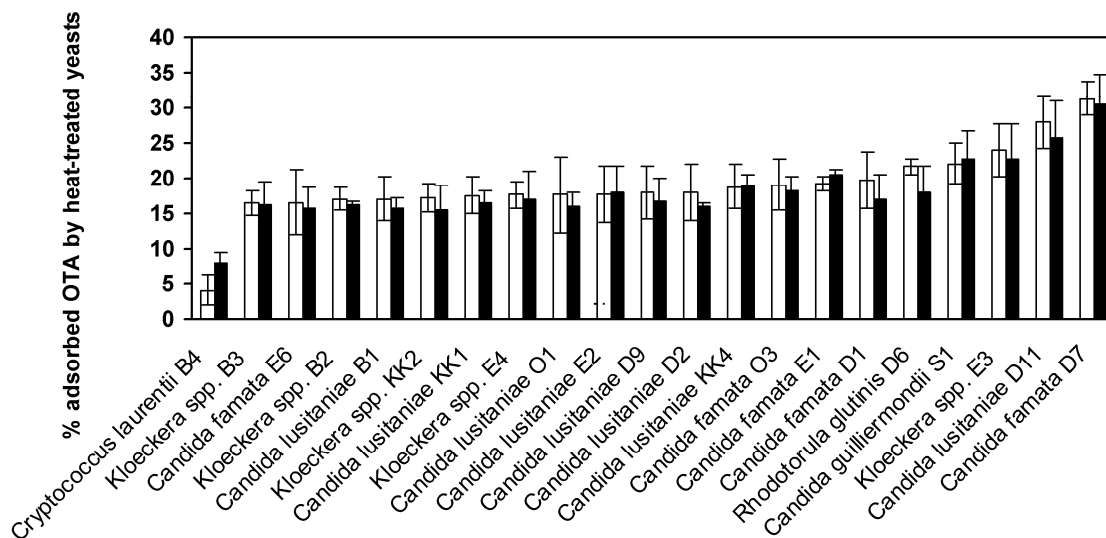


Fig. 2. Adsorption abilities of heat-treated yeasts isolated from Turkish wine-grapes within 4 h (in PBS, □; in white wine, ■).

tion ability of OTA by heat-killed yeast strains between PBS and white wine were not significant. On the other hand, heat treatment of the yeast cells enhanced their ability to remove OTA from PBS and wine. For instance; the adsorption of OTA (10 ng/mL) was 21.40% and 30.45% for viable and heat-killed *C. famata* D7, respectively in white wine.

These results clearly demonstrated that cell viability is not prerequisite for the removal of OTA from PBS and white wine. It is likely that the OTA molecule is bound to the surface of the yeast cells. This result confirms the preliminary observation by Bejaoui et al.¹, who found that heat-treated *Saccharomyces* strains showed higher adsorption (90%) when compared to viable cells (35%). Similarly, Péteri et al.¹⁵ observed that both viable and heat-treated (dead) *Phaffia rhodzyma* cells were able to adsorb significant amounts (up to 250 ng/mL) of OTA.

The adsorption ability of OTA by yeast strains was found to be strain specific. This may be due to the different cell wall structure of isolated yeasts. The cell wall, which is located outside the plasma membrane consists of two layers. The inner layer provides cell wall strength, and is made of β -1,3 and β -1,6-glucan that is complexed with chitin. The outer layer consists of mannoproteins, and determines most of the surface properties of the cell. The majority of the cell wall proteins (mannoproteins) are covalently linked to the inner glucan layer²⁰. Yeasts mannoproteins have been suggested to be an important element in the OTA removal assay, due to mycotoxin-binding capacity demonstrated for modified mannanoligosaccharide derived from the cell wall of *S. cerevisiae*³. As for other adsorption processes¹¹, the physical structure of mannoproteins—the total charge, charge distribution, the size of the pores and accessible surface area—play a significant role in the achievement adsorption differences among strains³. In several yeasts, including the *Saccharomyces*, the glycan portion of mannoproteins is composed not only of neutral oligosaccharides containing mannose and N-acetylglucosamine, but also of acidic oligosaccharides containing mannosylphosphate, in quantities which

vary from strain to strain. This structural variability may explain the differences in the binding ability of wine yeasts towards OTA and other components². The ability of the yeast cell wall-derived esterified glucomannan to bind AFB₁ in solution has been reported previously⁶. The cell wall polysaccharides (glucan and mannan), proteins and lipids have been reported to exhibit numerous different adsorption mechanisms, e.g., hydrogen bonding, ionic or hydrophobic interaction¹¹. Similarly, it has been observed that the β -D glucans isolated from *S. cerevisiae* cell wall is directly involved in the binding process of zearalene²¹.

CONCLUSIONS

Our results indicated that the adsorption ability of yeasts isolated from wine-grapes varied and yeasts could be a potential decontaminating agent for OTA in wine and other liquids without a lengthy incubation period. It has been also shown that yeast cell viability is not significant for the removal of OTA from a buffered solution and white wine. As the mechanisms of mycotoxin binding by yeasts are unclear in the literature, further studies regarding the chemistry of binding and the conditions releasing bound toxin molecules need to be investigated. Moreover, more yeast cells especially *S. cerevisiae* isolated from wine grapes should be tested to bind to OTA, and thus reduce exposure of humans and/or animals to these highly toxic contaminants in food.

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