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Adsorption of ochratoxin A and zearalenone by potential probiotic *Saccharomyces cerevisiae* strains and its relation with cell wall thickness

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Abstract

Aims: To examine *Saccharomyces cerevisae* strains with previously reported beneficial properties and aflatoxin B_1 binding capacity, for their ability to remove ochratoxin A (OTA) and zearalenone (ZEA) and to study the relation between cell wall thickness and detoxificant ability of yeast strains.
Methods and Results: A mycotoxin binding assay at different toxin concentrations and the effect of gastrointestinal conditions on mycotoxin binding were evaluated. Ultrastructural studies of yeast cells were carried out with transmission electronic microscopy. All tested strains were capable of removing OTA and ZEA. *Saccharomyces cerevisiae* RC012 and RC016 showed the highest OTA removal percentage, whereas RC009 and RC012 strains showed the highest ZEA removal percentages. The cell diameter/cell wall thickness relation showed a correlation between cell wall amount and mycotoxin removal ability. After exposure to gastrointestinal conditions, a significant increase in mycotoxin binding was observed.
Conclusions: All tested *Saccharomyces cerevisiae* strains were able to remove OTA and ZEA, and physical adsorption would be the main mechanism involved in ochratoxin A and ZEA removal. Gastrointestinal conditions would enhance adsorption and not decrease mycotoxin–adsorbent interactions.
Significance and Impact of the Study: Live strains with mycotoxin binding ability and beneficial properties are potential probiotics that could be included in animal feed. Previous and present results suggest that the RC008 and RC016 strains are very promising candidates for functional feed product development.

Introduction

In recent years, mycotoxin contamination of cereal grain and animal feed with mycotoxins has become a global concern. It is estimated that it may affect as much as 25% of the world's food crops each year. The most common mycotoxins found in animal feed are aflatoxins, ochratoxins, trichothecenes, fumonisins, zearalenone (ZEA) and ergot alkaloids (IARC 1993). Zearalenone is produced by *Fusarium* species, mainly by *F. graminearum* and *F. culmorum*, on a variety of cereal crops in temperate and warm regions of the world. This toxin and its derivatives are an important class of endocrine disrupters, which can cause oestrogenic effects and alterations in the reproductive tract of laboratory and domestic animals (WHO, 2000).

Ochratoxin A (OTA) is a potent mycotoxin with nephrotoxic, teratogenic and carcinogenic properties (Castegnaro *et al.* 1998). Based on sufficient evidence of carcinogenicity in experimental animals, OTA is

considered a possible human carcinogen (group 2B) (IARC 1993). Ochratoxin A and its hydroxyl derivatives are toxic when consumed by livestock (pigs, poultry) and humans (Ringot *et al.* 2007). This toxin is produced by two genera of fungi, *Aspergillus* and *Penicillium*, which grow on a variety of food and feed commodities during growth, harvest, storage and transportation (Bhatnagar *et al.* 2004). Ochratoxin A is mainly found in cereal and cereal products as well as in coffee, cocoa, wine, beer, pulses, spices, dried fruits, grape juice, pig kidney, and other meat and meat products of animal exposed to contaminated feedstuffs (Zöllner and Mayer-Helm 2006; Visconti *et al.* 1999; Meyvaci *et al.* 2005).

Concerns related to the negative health impacts of ZEA and OTA have led to the investigation of strategies to prevent their formation in foods, as well as to eliminate, inactivate or reduce this toxin bioavailability in contaminated products. Techniques to eliminate, inactivate or reduce the bioavailability of mycotoxins include physical, chemical and biological methods (Bata and Lásztity 1999; Heilmann *et al.* 1999; Belajová *et al.* 2007; Ringot *et al.* 2007). Physical methods involve extraction with solvent, adsorption, inactivation by heat and irradiation. Among the physical decontamination methods, adsorption on to various types of compounds (hydrated sodium calcium aluminosilicate—HSCAS, kaolin, silica binding agent, bentonite, etc.) has been extensively studied in recent years (Grant and Philips 1998; Scott 1998). Different binding agents including activated carbons (Galvano *et al.* 1998), zeolites (Tomasevic-Canovic *et al.* 2003) and diatomaceous earth (Natour and Yousef 1998) have been reported to remove OTA *in vitro*. Chemical methods, which alter the structure of mycotoxin by a chemical treatment (e.g. ammonia gas or ammonium hydroxide, 0·10% sodium hydroxide solution liquid, propionic acid solution bubbled with sulfur dioxide gas), have been used to convert mycotoxins to less toxic and mutagenic compounds or to immobilize them (Méndez-Albores *et al.* 2007). Limitations such as loss of product nutritional and sensory qualities as well as the expensive equipment required for these techniques have encouraged the recent emphasis on biological methods (Teniola *et al.* 2005).

An interesting approach for mycotoxin decontamination in feedstuff is the use of functional food that contains beneficial micro-organisms, which may bind mycotoxins in the gastrointestinal tract (GIT), thus reducing the extent of their absorption and systemic toxicity (Ramos and Hernandez 1996; Huwig *et al.* 2001). Yeasts have been shown to bind different mycotoxins strongly to their cell wall components. This mode of mycotoxin decontamination is highly promising. *Saccharomyces cerevisiae* strains, due in large part to their GRAS (Generally Regarded As Safe) status and probiotic use, are of particular interest for reducing the bioavailability of mycotoxins (Savage *et al.* 1996; Stanley *et al.* 1996; Kühle van der Aa *et al.* 2005).

Information concerning the mechanism of mycotoxin binding to cell wall is still scarce. However, a correlation between the β-D-glucan amount in the yeast cell wall and the efficacy of sequestering ZEA, aflatoxin B_1 (AFB_1), deoxinivalenol (DON), patuline (PAT) and OTA were demonstrated (Yiannikouris *et al.* 2004). On the other hand, several studies report that some fungal, bacterial and yeast strains were able to *in vitro* biodegrade AFB_1 (Motomura *et al.* 2003; Zjalic *et al.* 2006; Guan *et al.*, 2008) and OTA (Patharajan *et al.* 2011) and ZEA (Takahashi-Ando *et al.* 2005). This fact suggests that more than one mechanism of mycotoxin clearance could be involved. Studies regarding binding chemistry, especially under harsh conditions of GIT, need to be investigated. Changes in pH and the presence of bile, as important homoeostatic conditions that yeast cells encounter during passages through the GIT, are of particular interest.

In a previous study, the ability of *S. cerevisiae* isolated to adsorb AFB_1 and to tolerate the GIT was demonstrated (Armando *et al.* 2011). These strains were also able to retain mycotoxin binding ability under ruminant GIT conditions and improve ruminal fermentation (Dogi *et al.* 2011). However, no studies have evaluated the ability of these yeast strains to adsorb OTA and ZEA, and the influence of bile and low pH on this property as well as the possible mechanisms involved in mycotoxin clearance. The purpose of this study was to evaluate the ability of *S. cerevisiae* strains, obtained from feedstuff and pig gut, to remove OTA and ZEA under GIT conditions *in vitro* and the correlation between cell wall thickness and mycotoxin reduction.

Materials and methods

Yeast strains, growth medium and cultural conditions

Saccharomyces cerevisiae strains RC008, RC009, RC012 and RC016 were obtained from the collection centre at the Universidad Nacional de Río Cuarto, Argentina. They were previously isolated from feedstuff (RC008 and RC009 strains) and pig gut (RC012 and RC016 strains) and characterized by molecular techniques (Armando *et al.* 2011). Stock cultures were maintained at 80°C in 30% (v/v) glycerol. Working cultures were prepared from frozen stocks by two transfers in Yeast Peptone Dextrose broth incubation at 37°C for 24 h.

To conduct mycotoxin binding assays, inoculations were made from the working cultures into 30 ml of YPD broth and incubated for 24 h at 37°C. After being incubated, cells were collected by centrifugation (5000 ***g***,

10 min) and washed twice with phosphate-buffered saline (PBS, pH 7·2). Finally, the yeast pellets (1×10^7 cells ml^{-1}) were suspended in 20 ml of sterile PBS prior use for assays. The yeast pellet concentration was determined using a haemocytometer.

Ochratoxin A and zearalenone binding assay

The mycotoxin binding assay was performed according to Armando *et al.* (2011). Solutions of OTA (1, 5, 10, 40 and 100 μg ml^{-1}) and ZEA (1, 5, 10, 20 and 50 μg ml^{-1}) were prepared in PBS (pH 7·2). Yeast pellets, prepared as described earlier, were incubated for 1 h at 37°C in a shaking bath with 1 ml of each mycotoxin concentration in PBS, separately. Then, the cells were pelleted by centrifugation for 15 min at 5000 ***g*** at room temperature, and the supernatant containing unbound mycotoxin was collected and stored at −20°C for high performance liquid chromatography (HPLC) analysis. Positive (PBS + mycotoxin) and negative (PBS plus yeast cells) controls were included for all experiments. Three replications were carried out for each treatment, and the experiment was repeated twice.

The mycotoxin concentrations in which the maximum adsorption occurred most of the times were selected to study the GIT influence. Then, the concentrations 100 and 10 μg ml^{-1} for OTA and ZEA, respectively, were selected to carry out the further assays.

Effect of pH and bile on OTA binding

Ochratoxin A binding was tested under GIT simulated with pH 2 and bile salts (a mixture of conjugated bile salts, predominantly sodium glycocholate and sodium taurocholate along with a small amount of free bile acids -cholic acid- bile salts no. 3 Oxoid L56). Yeasts (10^7 cells ml^{-1}) were incubated for 4 h at 37°C in YPD broth (pH 7; control group); YPD broth (pH 2) or YPD with bile salts 0·5% (w/v) (pH 7). After incubation, cells were harvested and then incubated with 1 ml of OTA (100 μg ml^{-1}) in PBS for 1 h at 37°C in a shaking bath.

Cells were centrifuged for 15 min at 5000 ***g***, and the supernatant containing unbound mycotoxin was collected and stored at −20°C for HPLC analysis.

Effect of simulated gastrointestinal conditions on zearalenone binding

To improve the assay carried out previously for OTA binding, a simulated gastrointestinal passage was performed for ZEA binding. Yeast cells were resuspended in simulated gastric juice (NaCl, 125 mmol; KCl 7, mmol; $NaHCO_3$, 45 mmol; and pepsin, 3 g l^{-1}; adjusted to pH 3 with HCl) and incubated for 60 min at 37°C under agitation to simulate the peristalsis. After centrifugation, cells were added to artificial intestinal fluid (trypsin 1 mg ml^{-1}-Fluka 11531 U mg^{-1}; chymiotrypsin 1 mg ml^{-1}-Fluka 80 U mg^{-1}; oxgall bile salts 0·3% (w/v) in water and adjusted to pH 8 with NaOH 5 mmol l^{-1}) and were incubated for 60 min at 37°C under agitation. After that, yeast cells were washed twice with PBS and incubated with 1 ml of ZEA (10 μg ml^{-1}) in PBS for 1 h at 37°C in a shaking bath. Cells were centrifuged for 15 min at 5000 ***g***, and the supernatant containing unbound mycotoxin was collected and stored at −20°C for HPLC analysis.

Detection and quantification of ochratoxin A

Ochratoxin A was detected and quantified by reversed-phase HPLC according to Bragulat *et al.* (2001). The HPLC with fluorescence detection (λexc 330 nm; λem 460 nm) consisted in a C18 column (Supelcosil LC-ABZ 150 × 4·6 mm, 5 μm particle size; Supelco, Bellefonte, PA), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20 × 4·6 mm, 5 μm particle size). The mobile phase (57% acetonitrile: 41% water and 2% acetic acid) was pumped at 1·0 ml min^{-1}. The injection volume was 100 μl, and the retention time was around 5 min. The quantification limit of the technique was 0·1 ng g^{-1}.

Ochratoxin A binding percentage was calculated according to the following equation: 100 × [1 − (peak area of mycotoxin/peak area of mycotoxin in control)].

Detection and quantification of zearalenone

The used methodology was described by Cerveró *et al.* (2007). Extracts were resuspended in mobile phase methanol/water (70 : 30 v/v) and injected into the HPLC. Detection and quantification of ZEA was performed using a fluorescence detection system on Hewlett Packard 1100 Series chromatograph. The chromatographic separations were carried out on a C18 reverse phase column (150 × 4·6 mm, 5 μm particle size, Phenomenex, Luna, Torrance, CA), connected to a Supelguard LC-ABZ column (20 × 4·6 mm particle size, Supelco). The flow of mobile phase was 1 mL min^{-1}. The wavelength of excitation and emission used were 280 and 460 nm, respectively. The ZEA standards were prepared in acetonitrile (Sigma Aldrich, St Louis, MO, USA, purity >99%). The quantification limit of the technique was 3 ng g^{-1}.

Zearalenone binding percentage was calculated according to the following equation: 100 × [1 − (peak area of mycotoxin/peak area of mycotoxin in control)].

Recovery assay

Phosphate-buffered saline was spiked at three concentrations of OTA/ZEA (0·1, 0·2 and 10·0 ng ml^{-1}). After 1 h, samples were processed according to the above-mentioned procedure for extraction and estimation of OTA/-ZEA. Recovery experiments were performed in four replicates. Recoveries ranged from 89·2 to 90·7% for OTA and 87·6 to 92% for ZEA. The repeatability ranged from 2·51 to 2·87% for replicate analysis. The detection limit of the analysis was 0·02 ng OTA ml^{-1} and ng ZEA ml^{-1} of PBS medium.

Ultra-structural analysis of yeast cells

To elucidate some of the mechanisms involved in mycotoxin removal, yeast cells were analysed by transmission electronic microscopy (TEM). For TEM studies, *S. cerevisiae* strains were fixed in 2·5% glutaraldehyde in 0·2 mmol l^{-1} S-collidine pH 7·4, postfixed in 1% osmium tetroxide in 0·2 mmol l^{-1} S-collidine pH 7·4, dehydrated in increasing concentrations of acetone, embedded in EMbed 812 resin and sectioned on an ultramicrotome. These ultra-thin sections (60 nm) were cut and placed on copper grids, counterstained with saturated uranyl acetate and aqueous lead citrate. The sections were examined with transmission electronic microscope Elmiskop 101 (Siemens, Berlin, Germany). The yeast pellet was obtained by centrifugation after each step.

The measures of cell diameter and thickness of cell wall were obtained. Cell thickness/cell wall diameter relation was calculated for each yeast strain.

Statistical analyses

Mycotoxin binding levels at different concentrations, the effect of low pH and bile on mycotoxin binding and the relation cell diameter/cell wall thickness for each yeast strain were analysed by analysis of variance (ANOVA). Means were compared using the Fisher's protected LSD test (Quinn and Keough 2002). The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC, USA).

Results

Ochratoxin A binding by *Saccharomyces cerevisiae* strains

The ability of *S. cerevisiae* strains to remove OTA on *in vitro* conditions is summarized in Table 1. OTA binding percentages varied among yeast strains and with OTA concentration. All strains showed different mycotoxin binding ability according to different mycotoxin

Table 1 Ochratoxin A (OTA) and zearalenone (ZEA) binding levels (μg ml^{-1} and percentage %) by *Saccharomyces cerevisiae* strains at different toxin concentrations

Saccharomyces cerevisiae strains	Mycotoxin (μg ml^{-1} ± SD) and binding percentage (%)									
	1 μg ml^{-1} OTA	5 μg ml^{-1} OTA	10 μg ml^{-1} OTA	40 μg ml^{-1} OTA	100 μg ml^{-1} OTA	1 μg ml^{-1} ZEA	5 μg ml^{-1} ZEA	10 μg ml^{-1} ZEA	20 μg ml^{-1} ZEA	50 μg ml^{-1} ZEA
RC008	0·46 (46·0%) ± 0·07^{b}	0·80 (16·0%) ± 0·03^{b}	1·45 (14·5%) ± 0·03^{b}	7·17 (17·9%) ± 0·03^{b}	56·7 (56·7 %) ± 0·08^{b}	0·48 (48·0%) ± 0·02^{b}	2·83 (56·6%) ± 0·65^{c}	5·67 (56·7%) ± 1·10^{b}	12·35 (61·7%) ± 0·26^{a}	20·6 (41·1 %) ± 1·28^{b}
RC009	0·43 (43·0%) ± 0·05^{b}	0·80 (16·0%) ± 0·03^{b}	4·94 (49·4%) ± 0·09^{a}	14·95 (37·3%) ± 0·05^{a}	67·1 (67·2%) ± 0·09^{a}	0·85 (85·0%) ± 0·03^{a}	3·64 (72·8%) ± 0·08^{a}	6·72 (67·2%) ± ± 0·08^{ab}	12·81 (64·1%) ± 0·3^{a}	33·39 (66·8%) ± 3·84^{a}
RC012	0·63 (63·0%) ± 0·04^{a}	1·96 (39·2%) ± 0·07^{a}	5·64 (56·4%) ± 0·06^{a}	15·68 (39·2%) ± 0·05^{a}	71·2 (71·2%) ± 0·09^{a}	0·87 (87·0%) ± 0·02^{a}	3·90 (78·0%) ± 0·24^{a}	7·12 (71·2%) ± 0·45^{a}	10·41 (52·0%) ± 0·2^{a}	29·18 (58·4%) ± 0·58^{b}
RC016	0·74 (74·0%) ± 0·06^{a}	1·52 (30·4%) ± 0·03^{a}	5·80 (58·0%) ± 0·04^{a}	15·68 (39·2%) ± 0·05^{a}	74·2 (74·2%) ± 0·09^{a}	0·53 (53·0%) ± 0·02^{b}	3·03 (60·6%) ± 0·99^{b}	7·42 (74·2%) ± 0·39^{a}	10·35 (51·7%) ± 0·37^{a}	34·33 (68·7%) ± 0·62^{a}

Yeasts (10^{7} cells ml^{-1}) were incubated for 1 h at 37°C in a shaking bath with each OTA and ZEA concentration. Cells were centrifuged for 15 min at 5000 ***g*** and the supernatant containing unbound mycotoxin was collected and stored at −20°C for HPLC analysis.
Letters in common are not significantly different according to Fisher's protected LSD test ($P < 0·001$). Binding level (μg ml^{-1}) at each concentration of OTA was statistically analyzed separately (different letters indicate differences within each column).
Controls with each *Saccharomyces cerevisiae* strain separately (without mycotoxin) and control with OTA and ZEA at each concentration separately (without yeasts) were included.

concentration assayed. Adsorption percentage ranged from 46 to 74% at 1 μg ml^{-1} OTA. When 5 μg ml^{-1} OTA were used, the adsorption varied from 16 to 39·2%; it ranged from 14·5 to 58% at 10 μg ml^{-1} OTA, from 17·9 to 39·2% at 40 μg ml^{-1} OTA and from 56·7 to 74·2% at 100 μg ml^{-1} OTA.

The RC012 and RC016 strains showed the highest adsorption percentages (ranging from 30·4 to 74·2% for RC016 and from 39·2 to 71·2% for RC012 strain) at all tested concentrations of OTA ($P < 0·0001$), followed by RC009 strain. At 1 and 100 μg ml^{-1} concentrations, the highest OTA binding levels were observed.

Results for the influence of low pH and bile salts on OTA binding by yeast cells are shown in Table 2. Tested pH values and bile salt concentration were representative of those found in the mammalian gastrointestinal tract. Acid pH had significant effect on OTA binding ($P < 0·0001$); the highest binding levels were observed for RC008 and RC009. Incubation in presence of 0·5% bile salts significantly increased OTA binding only for *S. cerevisiae* RC008 and RC009 strains ($P < 0·0001$).

Zearalenone binding by *Saccharomyces cerevisiae*

The ability of *S. cerevisiae* strains to remove ZEA on *in vitro* conditions is shown in Table 1. As it was observed for OTA binding, ZEA binding percentages varied among yeast strains and with ZEA concentration. In general, all strains were able to adsorb the assayed ZEA concentrations. At the lowest ZEA concentrations (1 and 5 μg ml^{-1}), the binding percentage ranged from 48 to 87%, whereas at higher ZEA concentrations (20 and 50 μg ml^{-1}), the binding percentage ranged from 41·1 to 68·7%. *Saccharomyces cerevisiae* RC009 and RC012 strains showed the highest ZEA reduction percentages at the lowest concentrations (1 and 5 μg ml^{-1}) whereas RC016 showed the highest ZEA removal at 10 μg ml^{-1}. The reduction percentages obtained with RC009 strains ranged from 64·1 to 85%. It was observed that the removal percentages varied depending on the mycotoxin concentration levels.

Results for the influence of GIT conditions on ZEA binding by yeast cells are shown in Table 2. After exposure to GIT conditions, *S. cerevisiae* RC009 and RC016 significantly increased their ZEA binding ability ($P < 0·0001$).

Ultra-structural analysis of yeast cells

Morphology of the yeast cell wall determined by TEM is shown in Figure 1. The wall of *S. cerevisiae* is often described in terms of three layers, namely, an outer electron-dense layer, an adjacent less-dense layer and another dense layer that borders the plasma membrane, as can be observed in the TEM micrographs in Fig. 1. Mean cell wall values of each strain are related to the cell diameters ($n = 330$) and cell wall thickness ($n = 250$). Yeast cells exhibit great diversity with respect to diameter and wall thickness. The relationship between size and wall thickness shows an accurate estimation of cell wall content. So, the relation between them was evaluated to determine the proportion of cell wall present in each yeast strain, and the obtained data are shown in Table 3. There was not a correlation between diameter and cell wall thickness (data not shown). Moreover, the results showed that the strain RC016 had the lowest whole cell diameter. *Saccharomyces cerevisiae* RC016, RC012 and RC009 had thicker cell walls than RC008. Statistical analysis demonstrated that RC016

Table 2 Ochratoxin A (OTA) and zearalenone (ZEA) binding (%) by *Saccharomyces cerevisiae* strains previously treated with pH 2 and bile salts (0·5%) and after simulated gastrointestinal passage

	OTA binding levels (%) Media ± SD			ZEA binding levels (%) Media ± SD	
Saccharomyces cerevisiae strains	Control	pH 2	Bile salts (0·5%)	Control	After simulated gastrointestinal tract
RC008	56·7 ± 24·5^{b}	82·3 ± 6·02^{a}	74·4 ± 14·5^{a}	56·7 ± 11·0^{a}	59·19 ± 4·29^{a}
RC009	67·1 ± 21·27^{b}	80·2 ± 7·22^{a}	78·7 ± 12·34^{a}	67·2 ± 8·0^{b}	97·8 ± 1·48^{e}
RC012	71·2 ± 5·91^{a}	76·6 ± 4·51^{a}	72·1 ± 15·79^{a}	71·2 ± 4·5^{c}	77·2 ± 5·14^{c}
RC016	74·2 ± 4·74^{a}	75·9 ± 9·25^{a}	71·3 ± 1·33^{a}	74·2 ± 3·9^{c}	87·4 ± 3·84^{d}

Yeast (10^7 cells ml^{-1}) were initially incubated with YPD broth (pH 7) (control); YPD (pH 2) and YPD with bile salts 0·5% (pH 7), for 4 h at 37°C and then incubated with OTA (100 μg ml^{-1}) in OTA binding assay and yeast (10^7 cells ml^{-1}) were initially incubated in PBS (pH 7) at 37°C (control); PBS with salivary, gastric and intestinal conditions (Simulated gastrointestinal tract) and then incubated with ZEA (10 μg ml^{-1}) in ZEA binding assay. Cells were centrifuged for 15 min at 5000 ***g*** and the supernatant containing unbound mycotoxin was collected and stored at −20°C for HPLC analysis.

Letters in common are not significantly different according to Fisher's protected LSD test ($P < 0·001$). Binding level for each mycotoxin was statistically analyzed, separately.

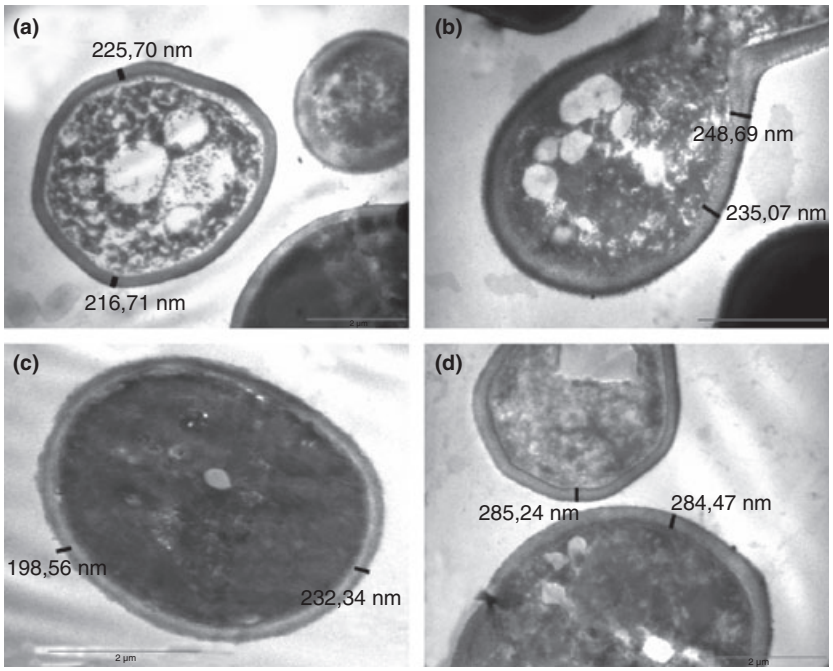

Figure 1 Ultrastructural analysis of yeasts cell: (a) RC008; (b) RC009; (c) RC012 and (d) RC016 strains. Magnification 12 000×. Mean cell wall values of each strain are for the cell diameters (n = 330) and cell wall thickness (n = 250).

Table 3 Ultrastructural analysis of *Saccharomyces cerevisiae* strains: relationship between cell wall thickness/cell diameter (μm)

S. cerevisiae strains	Diameter of whole cells (μm)*	Thickness of cell wall (μm)*	Cell wall thickness/cell diameter/(μm)**
RC008	4·21 ± 0·55[a]	0·375 ± 0·10[a]	0·08973920 ± 0·02479619[c]
RC009	4·18 ± 0·54[a]	0·419 ± 0·09[b]	0·10501329 ± 0·02836057[a,b]
RC012	4·55 ± 0·52[b]	0·429 ± 0·11[b]	0·09506561 ± 0·02555392[b]
RC016	3·98 ± 0·54[c]	0·438 ± 0·11[b]	0·11170665 ± 0·03306428[a]

Different letters indicate differences within each column, according to Fisher's protected LSD test *(P < 0·05); ** (P < 0·0001).

strain showed the highest relationship between the diameter and wall thickness, followed by RC009 strain, which was not significantly different from RC012. Strain RC008 showed the lowest relationship.

Discussion

Probiotics are a great promise for reducing the bioavailability of consumed mycotoxins. Several authors have demonstrated the safety of *S. cerevisiae* for nutritional and pharmaceutical use in animal feed in the European Union and Japan (Nitta and Kobayashi 1999). The Food and Drug Administration has given level of insurance or GRAS micro-organism degree to *S. cerevisiae* strains (http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm).

In this work, four strains of *S. cerevisiae* previously reported to be efficient AFB_1 adsorbents (Armando *et al.* 2011) were studied for their ability to bind OTA and ZEA. The results showed that all the tested strains were capable of binding OTA and ZEA at a greater or lesser extent. Similar results were reported by Bejaoui *et al.* (2004) who used *Saccharomyces* spp. for OTA (2 μg ml^{-1}) removal in grape juice. They showed an adsorption of 90% after 5 min exposure. However, Angioni *et al.* (2007) reported that cell walls of *S. cerevisiae* and *Kloeckera apiculata* do not adsorb OTA under *in vitro* interaction between OTA and yeast strains. A yeast-based product was reported to adsorb up to 68% ZEA, 29% AFB_1 and 62% OTA (Joannis-Cassan *et al.* 2011). However, these products and inorganic materials such as bentonites and aluminosilicates, used for mycotoxin adsorption, do not exert any beneficial properties. In previous works, RC008 and RC016 strains were selected because of their mycotoxin removal ability and probiotic effects such as showed coaggregation and antimicrobial

activity against all the bacterial pathogens isolated from clinical cases (*Escherichia coli*, *Enterobacter cloacae* and *Salmonella enterica* sub. sp. *Enterica*) (Armando *et al.* 2011) and improvement of ruminal fermentation (Dogi *et al.* 2011).

In this work, the relation between whole cell wall thickness and cell diameter was evaluated statistically ($P < 0{\cdot}0001$) to determine the proportion of cell wall present in each yeast strain. The relation cell wall thickness/cell diameter obtained for RC008 strain showed small cell wall content. This yeast strain was less efficient in mycotoxin removal, compared with RC009, RC012 and RC016 strains that showed the highest mycotoxin removal percentage and the greatest cell wall contents. These data suggest that the yeast-mycotoxin interaction is more of 'adsorption type'. Moreover, they showed that the greater cell wall content the greater ability to remove OTA and ZEA.

These biosorption mechanisms can be affected by environmental conditions such as pH, the presence of bile and temperature. Therefore, it is important to evaluate *S. cerevisae* strains for their ability to bind mycotoxin during passage through the GIT tract, considering low pH and bile presence.

In vitro adsorption assays may not always be indicative of the *in vivo* responses to specific mycotoxins; unfortunately, *in vivo* experiments are often very difficult to perform. A GIT model that reproduces physiological parameters, such as pH, peristaltic movements and gastric and intestinal secretions, is suitable for prescreening studies and may be a good alternative for *in vivo* experiments (Smeets-Peeters *et al.*, 1998). Few studies have assayed the ability to transit through simulated GIT conditions (Charteris *et al.* 1998; Chung *et al.* 1999; Liu *et al.* 2007). In this work, such exposure had a significant impact on OTA binding, enhancing mycotoxin adsorption. Similarly, after simulated GIT passage, an increase in ZEA binding ability by RC009 and RC016 strains was observed.

Hernandez-Mendoza *et al.* (2009) have recently reported that *Lactobacillus casei* strains subjected to bile salts were able to bind AFB_1.

Some studies have reported adsorption efficiency comparing the initial binding and then measuring desorption of the mycotoxin after exposure to a solvent system (Haskard *et al.* 2001). Nevertheless, this type of test does not represent the *in vivo* situation, where desorption in the GIT could occur. In this work, desorption assay was carried out with PBS at conditions found in GIT. The mycotoxin–yeast complex was found to be stable after gastrointestinal passage (data not shown).

This study reports, for the first time, the effect of simulated GIT conditions on mycotoxin adsorption and its influence on *S. cerevisiae* binding ability for OTA and ZEA. The conditions in the GIT tract would enhance adsorption and not decrease the mycotoxin–adsorbent interactions. The main mechanism involved in mycotoxin removal for these yeast strains was elucidated and is because of a physical adsorption; other studies could determine the influence on adsorption and complex formation in the cell wall, such as cell wall glucan and mannan content, as well as chitin content.

The importance of this work lays in the search for live strains to the further selection of potentially probiotic strains able to remove mycotoxin levels, to be included in a novel product for animal feed. In a previous work, RC008 and RC016 strains showed coaggregation and antimicrobial activity against all the tested pathogens (Armando *et al.* 2011) and improved ruminal fermentation (Dogi *et al.* 2011). Taken together, these results suggest that RC008 and RC016 strains are promising probiotic candidates to be included as feed additives. They are able to survive under GIT conditions, so they could exert their beneficial properties and they can also improve animal performance by reducing the bioavailability of AFB_1, OTA and ZEA at gut level.

Functional and technological tests should be performed to validate these strains as suitable probiotics for animals.

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