

Original Article**In Vitro Binding Potentials of Bentonite, Yeast Cell Wall and Lactic Acid Bacteria for Aflatoxin B₁ and Ochratoxin A**

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ABSTRACT

Background: This study intended to assess individual and combined adsorption potentials of three adsorbents (processed bentonite as an inorganic adsorbent, and cell walls of *Saccharomyces cerevisiae* and of the GG strain of *Lactobacillus rhamnosus* as organic adsorbents) for aflatoxin B₁ and ochratoxin A under in vitro conditions.

Methods: This study was conducted in Ferdowsi University of Mashhad, Mashhad, Iran in 2106. A pre-test with four treatments of bacteria and yeast (live or dead) with five replications was designed and conducted to study the effects of these biotic and abiotic organic adsorbents on toxin adsorption efficiency. The experiment in the main study had seven treatments including two toxins (2 ppm) and three adsorbents with five replications using the completely randomized design. Toxin quantities were measured by an HPLC instrument.

Results: The various types of dead organic adsorbents were more capable of adsorbing toxins compared to the live ones. The processed bentonite was considerably more efficient in adsorbing aflatoxin B₁ (93.51) compared to the other treatment groups ($P < 0.05$). No significant differences were observed between the effects of yeast and bacterial cell walls in adsorbing aflatoxin B₁. However, the bacterial cell walls (61.71) had higher adsorption efficiencies in adsorbing ochratoxin A than the processed bentonite and yeast cell walls ($P < 0.05$).

Conclusion: Processed bentonite (or montmorillonite) and bacterial cell walls are able to adsorb considerable quantities of aflatoxin B₁ and ochratoxin A, and can be used in multiple mycotoxin contaminations as an effective strategy for preventing or reducing the harmful effects of these toxins.

Keywords: Adsorbents, Bentonite, Detoxification, *Lactobacillus*, Mycotoxin, Yeast Cell Wall.

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INTRODUCTION

The Food and Agriculture Organization (FAO) has estimated that about 25% of global agricultural products are contaminated with mycotoxins causing economic losses and health harms. Depending on the type of mycotoxins, eating meals or foodstuffs contaminated with them can cause problems like lack of optimal fetal growth and development, cancer, estrogenic effects, nervous system destruction, and immune system suppression in humans and animals [1]. Mycotoxins are secondary metabolites produced by many fungi especially those of *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* genera [2]. The most important mycotoxins with respect to general health and agriculture are aflatoxins, ochratoxins, trichothoxins, fumitoxins, and zearalenone. Aflatoxins, the most common and hazardous metabolites that are produced by various *Aspergillus* species such as *A. flavus* and *A. parasiticus*, damage liver cells, reduce

milk and egg yields in livestock and poultry, suppress the immune system and, eventually, lead to substantial reductions in livestock performance [3]. Among the various members of the aflatoxin family, aflatoxin B₁ (AFB₁) has the maximum toxicity and carcinogenicity for humans and animals [4]. About five billion people in various countries are exposed to the hazard of AFB₁ through various contaminated animal food products [5]. Another common mycotoxin in nature is ochratoxin A (OTA) mainly produced by *A. ochraceus*, *A. bonarius*, and *Penicillium verrucosum*. OTA is one of the best-known agents harm cells in the hepatic system leading to liver diseases. Moreover, OTA causes cancer, suppresses the immune system, and destroys liver cells [6].

Considerable efforts have been made to eliminate mycotoxins from animal feeds including thermal inactivation, irradiation, microbial decomposition, and treatment with chemicals. Nevertheless, most of

these methods are impractical, ineffective, or costly under field conditions and may decrease yield animal and reduce food quality [7]. Clay soil-based adsorbents are one of the most efficient adsorbents for adsorbing AFB₁ [8]. Among the various clay soil adsorbents, bentonite and its main mineral montmorillonite have received special interest. The layer structure of montmorillonite allows it to swell when it is placed in a liquid environment, and it can thus adsorb AFB₁ on its layers and prevent adsorption of AFB₁ molecules by cells in the digestive system [9]. The various possible mechanisms by which clay soils may adsorb AFB₁ molecules are studied [10]. These mechanisms mostly act by forming chemical bonds between the active sites in AFB₁ (the β-D-dicarbonyl groups) and clay soils. The clay soils, especially montmorillonite, have useful effects on AFB₁ adsorption by the digestive system in livestock and poultry and can neutralize its harmful effects on health and yield of animals [9]. Nevertheless, this group of adsorbents is of low efficiency in adsorbing other toxins such as OTA [11].

Organic adsorbents, especially mannan-oligosaccharides isolated from cell walls of *S. cerevisiae* and also of lactic acid producing bacteria, appear to have considerable ability in adsorbing AFB₁ and other mycotoxins, too [12]. β-D-glucan and glucomannan in yeast cell walls were able to bind other mycotoxins especially OTA and zearalenone [13]. Furthermore, OTA removal by yeast cell walls is a physical adsorption process [14]. Lactic acid bacteria have been used for centuries in fermented foods, and many strains of LAB can bind aflatoxin [15, 16]. The GG strain of *L. rhamnosus* had the highest efficiency in adsorbing AFB₁ [16]. Moreover, these bacteria can adsorb more than 50% of the OTA content in aqueous media [17]. However, it is not completely clear yet whether dead bacteria through physical adsorption carry out this process by cell walls or live bacteria are able to reduce mycotoxin contents through production of toxin-decomposing enzymes. Therefore, removal efficiency and mycotoxin reduction by dead and live bacteria should be compared.

Consequently, reduction efficiencies of AFB₁ and OTA by dead and live bacteria were studied in a pre-test in the present research. The experiment in

the main research intended to study the abilities of two organic adsorbents (cell walls of *S. cerevisiae* and of the GG strain of *L. rhamnosus*) and an inorganic adsorbent (bentonite or montmorillonite) used alone or in combination in adsorbing AFB₁ and OTA.

MATERIALS AND METHODS

Adsorbents

The three adsorbents used in the pre-test and the main experiment included the inorganic sodium bentonite or processed montmorillonite (G. Bind, produced at the PayaFarayand Hezareh Novin Factory in Mashhad, Khorasan Razavi Province, Iran, in 2016). Sodium was entered into montmorillonite layers to activate the clay soil, and the clay soil was processed to improve its physical properties). Table 1 presents a chemical analysis of the processed sodium bentonite used in the present research.

One of the organic adsorbents were A18 *S. cerevisiae* cell walls (produced by the Microbiology Department, Khorasan Razavi Science and Technology Park). Potato dextrose agar was used first for mass culture and then, using a spectrophotometer at 600 nm and measuring optical density, the concentration of the yeast was found to be 2×10^8 /ml. Centrifugation at 3000 rpm was used to separate the solid phase from the liquid solution. It was washed in three stages using sterilized physiological serum [18]. Yeast suspensions were prepared in 0.1 M phosphate buffer solution and autoclaved at 120 °C or 20 min [15]. Finally, centrifugation at 5000 rpm was carried out to separate cell walls from the cytoplasmic extract and the precipitated cell walls were washed with sterilized distilled water. The other organic adsorbent, cell walls in the GG strain of *L. rhamnosus*, was produced in freeze-dried format at -40 °C. The bacteria were prepared by inoculating 100 ml of MRS Broth (De Man, Rogosa, Sharpe) (Oxoid, UK) by 1 gr of the culture and were put in an incubator at 37 °C or 20 h. One ml of this culture was then transferred to 99 ml of MRS Broth medium to produce a 1% dilution and was incubated again at 37 °C for 20 h.

Table 1. Chemical analysis (%) of processed sodium bentonite examined by X-ray fluorescence (XRF).

	SiO ₂	Al ₂ O ₃	Fe ₂ O ₃	MgO	CaO	P ₂ O ₅	Na ₂ O	K ₂ O	MnO	SO ₃	L.O.I ²
G.Bind™ ¹	65.14	10.34	2.26	2.10	2.66	0.06	2.67	0.30	0.41	0.72	12.34
1. Basic processed bentonite.											
2. Loss of ignition.											

As in the case of the yeast, bacterial concentration was also determined using a spectrophotometer at 600 nm and employing optical density measurements. The desired bacterial concentration in this research was 10^9 CFU/ml. The bacteria were incubated in the next stage together with 4 ml of 2M hydrochloric acid at 37 °C for 1 h to inactivate them and separate their cell walls. They were then washed twice with 4 ml of phosphate buffer solution. Finally, the sample was centrifuged at 5000 rpm for 10 min at less than 10 °C, the supernatant was removed before applying the mycotoxin treatment, and freeze-dried powders of both the yeast and of the bacteria were prepared.

Toxins and Their Adsorption Tests

Both AFB₁ and OTA with 99% purity were obtained from the Sigma Chemical Company (St. Louis, Mo, USA), and procedures described by Ledoux and Rottinghaus were followed to study efficiencies of toxin adsorption [19]. Using methanol, the main solution was first prepared at 1000 µg/ml. This solution was then used to prepare working solutions at 20 µg/ml, and the working solutions were diluted using phosphate buffer solution to achieve the final concentrations of 2 µg AFB₁/ml and 2 µg OTA/ml. Next, 10 mg/ml solutions of each adsorbent, both individually and in combination with the other adsorbent, were prepared and poured into propylene tubes with screw caps which toxin already contained toxins (there were 10 ml of the buffer solution, of the toxin, and of the adsorbent in each test tube). The seven treatment groups included three adsorbents (one inorganic and two organic adsorbents) individually and in combination, and each treatment was applied in three replications. The pH of the medium was maintained at about 6 to be close to the conditions in the duodenum. The 70 samples were then placed in a shaker incubator with shaking speed of 40 cycles per min at 37 °C for 30 min. They were finally centrifuged at 25 °C and 3000 rpm for 5 min, and 1 ml of the supernatant was removed from each tube for HPLC. In addition to the experimental groups, two tubes contained only AFB₁ and OTA separately and were used as the standard solutions in HPLC. Adsorption percentage (that is, adsorption efficiency) was calculated by comparing the initial concentrations of AFB₁ and OTA with their concentrations in the presence of the adsorbents using the following formula:

$$\left(1 - \frac{\text{Concentration of the toxin in the presence of the adsorbent}}{\text{Concentration of the toxin in the standard sample}}\right) \times 100$$

The method [20] was used employing an HPLC instrument to determine the toxin content of the samples. In short, 10 µl of the sample was injected to HPLC with 5 µm Supercoil TMLC-18 reverse phase column (250 × 4.6 mm, Supelco, Bellefonte, PA) and water: methanol: acetyl mixture (1:3:6 ratio) as the liquid phase with the flow rate of 1 ml/min, and toxin concentrations were measured using the maximum measured height.

Statistical Analysis

All data was analyzed using the general linear model employing SAS based on completely randomized design (with four treatments of live or dead bacteria and yeast and 5 replications for each treatment in the pre-test, and 7 treatments in the experiment of the main study that included bentonite, yeast and bacteria (individually or in combination) and 5 replications for each treatment. ANOVA was performed using Tukey's Honestly Significant Difference (HSD) test, and the difference between the means was compared when *P*-value was less than 0.05.

RESULTS

A pre-test was performed to study and compare the abilities of live and dead *L. rhamnosus* and *S. cerevisiae* in adsorbing AFB₁ and OTA (Table 2) (Fig. 1). The dead forms of the two organic adsorbents (the yeast and the bacteria) used in this study had higher efficiencies than the living forms in adsorbing AFB₁ (73.03 and 75.28% compared to, 44.23 and 46.71%, respectively) and in adsorbing OTA (45.53 and 61.71% compared to 34.43 and 40.06%, respectively). Therefore, the dead forms were also used in the main study.

Adsorption efficiencies of AFB₁ and OTA by the three various adsorbents (the inorganic adsorbent, processed bentonite, and the organic adsorbents, cell walls of *S. cerevisiae* and *L. rhamnosus*) were evaluated. Table 3 presents results related to the efficiencies of individual and combined use of the adsorbents in adsorbing AFB₁ and OTA under in vitro conditions. The group in which only the processed bentonite was used as the adsorbent was considerably more efficient than the groups with the yeast and bacterial cell walls (93.51% versus 70.03% and 75.28%, respectively). Furthermore, efficiency in AFB₁ adsorption when the adsorbents were poured in combination into the AFB₁ containing tubes in groups, which included the processed bentonite, was significantly higher compared to the group that only contained the combination of the bacteria and yeast. The combination of all three adsorbents led to the highest AFB₁ adsorption (94.50%), but the difference was not statistically significant (Table 3).

Table 2. Percentage of aflatoxin B₁ (AFB₁) and ochratoxin A (OTA) binding by live or dead *S. cerevisiae*¹ and *L. rhamnosus*² strain GG.

	AFB ₁		OTA	
	alive	dead	alive	dead
Yeast	44.45	73.35	34.52 ^b	45.45 ^b
Bacteria	46.46	75.52	40.55 ^a	61.43 ^a
SEM	0.58	0.68	1.12	2.69
P-value	0.085	0.117	0.0005	<0.0001

1. Yeast, 2. Bacteria

a-b: The means in each row with dissimilar letters are significantly different ($P < 0.05$); SEM = Standard deviations of the means

Table 3. Adsorption percentages of AFB₁ and ochratoxin A by the processed montmorillonite¹ and by cell walls of *S. cerevisiae*² and of the GG strain of *L. rhamnosus*³.

Treatments	Bentonite	Yeast	Bacteria	Bentonite + yeast	Bentonite + bacteria	Yeast + bacteria	Bentonite + yeast + bacteria	SEM	P-value
Aflatoxin B ₁	93.51 ^a	73.03 ^b	75.28 ^b	91.31 ^a	92.10 ^a	73.32 ^b	94.50 ^a	1.82	< 0.001
Ochratoxin A	48.07 ^c	45.53 ^c	61.71 ^{ab}	47.44 ^c	66.49 ^a	56.72 ^b	64.83 ^b	1.95	<0.001

¹Bentonite, ²Yeast, ³Bacteria

a-b: The means in each row with dissimilar letters are significantly different ($P < 0.05$); SEM = Standard deviations of the means

In general, the inorganic and organic adsorbents adsorbed less OTA compared to AFB₁. Montmorillonite and yeast cell walls exhibited the minimum efficiencies in adsorbing OTA when they were used individually or in combination. Although LAB was individually capable of adsorbing significantly more OTA (61.71%) than *S. cerevisiae* and the processed bentonite, yet OTA adsorption efficiency improved and reached 66.49% when LAB was used together with the processed bentonite, and 64.83% when they were applied in combination with the yeast. However, these differences were not statistically significant (Table 2).

DISCUSSION

Aflatoxins and OTA are classified as secondary carcinogenic metabolites and, therefore, their presence in meals and foodstuffs can be a threat to health of humans and animals. Many adsorbents are able to adsorb, bind, and inactivate various mycotoxins under in vitro conditions [21]. Consequently, the present study intended to assess three adsorbents (sodium bentonite, cell walls of *S. cerevisiae* and the GG strain of *L. rhamnosus*) with respect to adsorption of aflatoxin B₁ and OTA under in vitro conditions. Results of the pre-test indicated the dead bacterial and yeast cells were more efficient in adsorbing the toxins, which suggested that the studied toxins were physically adsorbed by these adsorbents. These results agree

with the findings of other researchers [2, 13, 14]. The process of mycotoxin removal by yeast or bacteria was carried out more with the help of physical adsorption than through enzymatic activity of the activated types of these microorganisms, and killing these microorganisms and breaking down their cell walls would create greater surface area for mycotoxin adsorption, especially for adsorption of polar mycotoxins.

The cell wall in *S. cerevisiae* includes a network of the main column (β -1, 3-glucan) and of lateral chains of β -1, 6-glucan bound to glycosylated mannan-proteins that form its outer layer [22]. The proteins and the glucans provide available adsorption sites capable of adsorbing toxins through various mechanisms such as hydrogen bonds and ionic or hydrophobic reactions. This yeast strain was highly capable of aflatoxin adsorption, and yeast cells killed by autoclaving had greater ability than the live form in adsorbing toxins [2]. Heat increases permeability of the outer layer of cell walls and may result in dissolution of cell-surface mannan and in increased adsorption regions. Adsorption of toxins by this adsorbent is a physical rather than a metabolic process. Other researchers also attributed aflatoxin adsorption by yeast cell walls to mannan oligosaccharides [13]. Nevertheless, research that is more precise is needed on intact cells and separate cell walls to better understand the adsorption process. Toxin adsorption by these bacteria is a physical process

because live bacteria were less able to adsorb AFB₁ than bacteria treated with hydrochloric acid. The mechanism of toxin removal by bacteria shows that AFB₁ molecules attach themselves to polysaccharides and peptide glycans present on bacterial cell walls.

Findings of the present study indicated that the processed sodium bentonite with the efficiency of more than 90% was more efficient in AFB₁ adsorption and that it could be claimed to be a suitable choice for studies under in vivo conditions. Results found by other researchers who used sodium bentonite or calcium montmorillonite [11] conform to those of the present research. Montmorillonite was first used in 1978 for separating aflatoxin and for reducing its toxicity or in vitro media [23]. Three different parts on the surface of montmorillonite (the outer basal planes and the interlayer edges and spaces) can adsorb aflatoxin molecules, and the interlayer spaces are the most important part for adsorption of aflatoxin molecules [7]. Two carbonyl groups in aflatoxin molecules have positive charges and are active regions that determine how capable toxin molecules are in binding to adsorbents.

Various suggested mechanisms indicate how aflatoxin molecules are adsorbed by montmorillonite: the electron donor-acceptor model, selective chemical adsorption, and hydrogen bonds. In the electron donor-acceptor model, the positive charges on aflatoxin molecules probably share electrons with negative charges present on montmorillonite surfaces and this leads to toxin adsorption by montmorillonite [7]. In the conceptual selective chemical adsorption model, enthalpy of the reaction between aflatoxin molecules and montmorillonite shows that the active carbonyl regions in aflatoxin can form chelates with transition metals in montmorillonite and thus separate the toxin from the medium [24]. The last probable mechanism is the formation of hydrogen bonds between toxin molecules and montmorillonite cations in interlayer spaces. The most important model in liquid media for describing toxin separation by clay minerals is the formation of hydrogen bonds between the active regions of toxin molecules and montmorillonite [10]. Moreover, exchangeable cations could be substituted to change montmorillonite capacity for adsorbing aflatoxin molecules [25].

In the present research, the processed bentonite or montmorillonite called G. BindTM was used in the processing of which sodium cations were added to the clay soil to stabilize sodium ions in the

interlayer spaces and thus increase their charge density in these spaces, which could increase adsorption power for aflatoxin molecules. Although the cell walls of *S. cerevisiae* and of the GG strain of *L. rhamnosus* were less efficient than the processed bentonite in adsorbing AFB₁, yet they were able to adsorb more than 70% (75.28%) of the AFB₁ toxin. The GG strain of *L. rhamnosus* could adsorb close to 80% of the AFB₁ in liquid media [20]. The higher efficiency of AFB₁ adsorption by the processed montmorillonite used alone or in combination groups that included this type of bentonite could be due to increased capacity of ion exchange between this product and AFB₁ molecules, to increased water absorption capacity, and to improved swelling index. Performing basic processing on montmorillonite causes expansion of interlayer spaces and thus helps montmorillonite to adsorb more easily greater quantities of AFB₁.

The adsorbents used in the present research were less efficient in adsorbing OTA than AFB₁. This could be somewhat due to the structural differences between these two toxins. An L-beta phenylalanine group is bound to OTA molecule. In the experiment conducted for the main study, bentonite and yeast cell walls were least able to adsorb OTA. In another study, processed zeolite was used for OTA adsorption under in vitro conditions at pH of seven. This product was able to adsorb up to 71% of the OTA [26], a higher efficiency than that in the present research (48.07%). Sodium-calcium aluminosilicates (clay soils) had low efficiencies in adsorbing OTA [11]. The percentage OTA adsorption by manna oligosaccharides in yeast cell walls (45.53%) was less even compared to bentonite. Adsorption by organic adsorbents results from the creation of bipolar charges, which suggests that efficiencies of such adsorbents heavily depend on the polar feature of mycotoxins [27]. There is very little information on OTA adsorption by yeast cell walls, but OTA adsorption by cell walls under in vitro conditions is only 12.5% [28]. Furthermore, mannan oligosaccharides in yeast cell walls were able to adsorb only 30% of the OTA [12]. The GG strain of *L. rhamnosus* had a higher efficiency (61.71%) than the other two adsorbents in adsorbing OTA. OTA adsorption increased (and reached 64.83%) when all three adsorbents were employed, but this increase was not statistically significant. The most important mechanism for OTA adsorption by LAB is through their cell walls. For example, *L. rhamnosus* was treated with heat and acid and OTA adsorption efficiency in phosphate buffer media by these bacteria was

higher compared to the live bacteria [29]. In their experiment, these bacteria were able to adsorb about 70% of the OTA in the phosphate buffer environment.

CONCLUSION

The processed bentonite could increase cation exchange in interlayer spaces of bentonite, improve the ability of this product in separating AFB₁ from the medium, and thus reduce their toxic effects. Moreover, the GG strain of *L. rhamnosus* exhibited the maximum efficiency in adsorbing OTA. Finally, since in most cases we witness multi-mycotoxin contaminations, a combination of processed bentonite and inactivated cell walls from the GG strain of *L. rhamnosus* may be the best choice for separating toxins such as AFB₁ and OTA from media. More research is required to understand better adsorption mechanisms related to mycotoxin adsorption by various organic and inorganic adsorbents and to determine their effects under in vivo conditions.

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