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In vitro ability of *Saccharomyces cerevisiae* and *Lacticaseibacillus rhamnosus* to bind aflatoxin B1 in phosphate-buffered saline solution

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Abstract

The utilization of strains of probiotic microorganisms has been demonstrated to be effective in the removal of mycotoxins. This study aimed to evaluate the *in vitro* adsorption capacity of aflatoxin B_1, by *Lacticaseibacillus rhamnosus* and *Saccharomyces cerevisiae*, either alone and in combination, as well as the stability of the adsorbent/mycotoxin complex in each of the situations. Aflatoxin B_1 working solutions for the assays were prepared in potassium phosphate buffer at pH 3.0 and pH 6.5, using aliquots of lactic acid bacteria and yeast culture containing a biomass of inactivated cells, either alone or in combination. After incubation, the solution was centrifuged, and the supernatant was separated for aflatoxin B_1 quantification. The stability test of the aflatoxin B_1 complex formed with all the mentioned treatments was carried out using the pellets obtained after centrifugation of the binding assays, performing multiple washes with phosphate buffer. The supernatant from each wash was analyzed for the amount of aflatoxin B1 released. The best aflatoxin B1 adsorption rate occurred with the use of lactic acid bacteria/*S. cerevisae* at both pH 3.0 (61.9%) and 6.5 (68.1%), followed by *S. cerevisiae* isolated at pH 3.0 (57.4%). Regarding the stability of the adsorbent/mycotoxin complex, they showed greater stability to *S. cerevisae* isolated at pH 3.0 (85.7%) and less stability to lactic acid bacteria at pH 3.0 (56.2%).

Keywords: AFB_1; *Saccharomyces cerevisiae*; *Lacticaseibacillus rhamnosus*; adsorption; decontamination.

Practical Application: The application of microbial products represents a promising avenue for the remediation of mycotoxin contamination.

1 INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi, the main producers being those of the genera *Aspergillus*, *Penicillium*, and *Fusarium*, and are found in food and animal feed. The Food and Agriculture Organization (FAO) estimates that about 25% of food is contaminated with mycotoxins. The ingestion of mycotoxins may cause mutagenicity, carcinogenicity, and teratogenicity, in addition to having immunosuppressive action. Exposure to mycotoxins can be direct, when ingesting contaminated grains, or indirect, when ingesting contaminated animal products. An example of indirect consumption is contamination by mycotoxins in milk and its derivatives. Mycotoxins ingested by ruminants are metabolized by the ruminal microbiota and excreted in milk. Milk and its derivatives are an important source of nutrients for growing children. Studies point to a relationship between aflatoxin B_1 (AFB_1) exposure and problems in fetal and infant development (Taheur et al., 2017).

Several strategies have been used to reduce or eliminate the effects of mycotoxins, including inhibition of mold growth in food, detoxification of food and feed, and reduction of mycotoxin absorption in the gastrointestinal tract. The use of adsorbent agents with the ability to bind to mycotoxins in the animal digestive tract has been shown to cause a reduction in the bioavailability and toxicity of mycotoxins. Among these adsorbent agents, strains of probiotic microorganisms have been shown to be effective in removing mycotoxins, especially the yeast *Saccharomyces cerevisiae* and the lactic acid bacteria (LAB) *Lactobacillus acidophilus* (Afshar et al., 2020).

Studies evaluating the ability of LAB and *S. cerevisiae* to remove AFB_1 indicated a rapid binding process between AFB_1 and the microorganisms. The binding occurred through the formation of a reversible complex between the toxin and the surface of the microorganism, with no chemical modifications of the mycotoxin. AFB_1 adsorption levels depend on the concentration of both the mycotoxin and the microorganisms, with comparable adsorption observed using either viable or non-viable cells (Bueno et al., 2007).

S. cerevisae has been shown to be effective in binding AFB_1 and aflatoxin M_1 (AFM_1) in ultra-high-temperature (UHT) skim milk, due to the composition of the cell wall of *S. cerevisiae*, which is composed of a bilayer structure of β-1,3-glucan and β-1,6-glucan. It appears that its cell surface has several sites for physical adsorption. Removal of aflatoxins occurs through

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adsorption to cell wall components and not through covalent binding or metabolic degradation, given that the binding capacity is maintained in dead cells after heat treatment. Both removal and release of aflatoxin are processes that occur quickly and reversibly, with no chemical modification of AFB_1. The ability to remove AFB_1 will depend on the concentration of toxins and the amount of bacteria used. The process is therefore reversible and fast in nature, characterized by a physical adsorption (physisorption), which involves weak Van der Waals binding forces, hydrogen bonds, and hydrophobic interactions (Bueno et al., 2007; Karazhiyan et al., 2016).

LAB is a probiotic used in foods and considered safe in fermented foods, and has been used to decontaminate heavy metals, pesticides, and mycotoxins. LAB binds to aflatoxins through a weak, non-covalent, and reversible cell wall-mediated interaction. The binding capacity is affected by several factors such as pH, temperature, strain type, strain concentration, and aflatoxin concentration. The *in vitro* adsorption of aflatoxins by LAB shows high efficiency, demonstrating greater adsorption capacity after heat treatment, with an increase from 54 to 81 to 86% in adsorption rates (Liu et al., 2020; Luo et al., 2020).

Through the use of new biotechnologies, microbial products have emerged as promising tools in the decontamination of mycotoxins. They help reduce the problems caused by fungi in the food chain, as well as offer new approaches, such as the combination of different probiotics, in the search for better results in the degradation and adsorption of mycotoxins. This work brings new information about the adsorption capacities and stability of the adsorbent/mycotoxin complex by probiotics to help us obtain useful information in the selection of more efficient adsorbents, as well as to clarify the adsorption relationships, enabling a future industrial application of these resources. The objective of this work was to evaluate the in vitro adsorption capacity of AFB_1, by *Lacticaseibacillus rhamnosus* and *S. cerevisiae*, either alone and in combination, as well as the stability of the adsorbent/mycotoxin complex in each of the situations.

1.1 Relevance of the work

The application of novel biotechnologies is transforming microbial products into promising tools for the decontamination of mycotoxins, thereby mitigating the adverse effects of fungi in the food chain. Additionally, these technologies are facilitating the exploration of innovative approaches, such as the combination of diverse probiotics, with the aim of achieving enhanced outcomes in the degradation and adsorption of mycotoxins. This study contributes new insights into the adsorption capacities and stability of the adsorbent/mycotoxin complex by probiotics, facilitating the selection of more efficient adsorbents and elucidating adsorption relationships, thereby advancing the potential industrial application of these resources.

2 MATERIAL AND METHODS

2.1 Preparation of lactic acid bacteria and yeast strain biomass

A commercially available lyophilized *L. rhamnosus* HOWARU® LYO 40 DCU containing 1.0×10^{10} cells/g, kindly donated by Danisco Brazil Ltda, was used in the experiment. This strain was previously evaluated in the Mycotoxin Control and Decontamination Laboratory of the School of Animal Science and Food Engineering of the University of São Paulo (LCDM/ZEA/FZEA-USP) located in Pirassununga, SP, Brazil, regarding its ability to bind, with percentage bindings of 36–46% (Bovo et al., 20134). The *S. cerevisiae* strain was a commercially available brewer's biological dry yeast (Fermentis K-97, SafAle, Bruggeman, Belgium) containing 1.0×10^{10} yeast cells/g, previously evaluated in LMMA, showing high capacity (90–93%) to bind to AFM_1 (Corassin et al., 2013). Both the *L. rhamnosus* strain in the lyophilized starter culture and *S. cerevisiae* cells were inactivated by autoclaving the material at 121°C for 10 min.

2.2 Aflatoxin Adsorption

AFB_1 working solutions for the assays at 1.0 µg/mL were prepared in 0.1 M potassium phosphate buffer (PBS) at pH 3.0 and pH 6.5, as described by Campagnollo et al. (2015). The procedures will also follow the recommendations proposed by Joannis-Cassan et al. (2011) for standardized adsorption study methods, including preliminary tests to ensure that mycotoxins in buffer solutions were not degraded or absorbed on tube walls during the adsorption tests. One-gram aliquots of the LAB and yeast culture (containing a biomass of 1×10^{10} inactivated cells), alone or in combination, were transferred to triplicate test tubes containing 1.5 mL of AFB_1 buffer solutions (pH 3.0 and 6.5) and placed on a rotating shaker at 180 rpm for 60 min at room temperature (25°C). After incubation, the solution was centrifuged at 3,100 × g for 10 min, and the supernatant was separated for quantification of AFB_1 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Negative controls (*L. rhamnosus* and/or *S. cerevisae* cells suspended in buffer solution) and positive controls (AFB_1 in buffer solution) were also incubated and analyzed as described. The percentage of mycotoxin bound was calculated using Equation 1, where A is the percent of AFB_1 adsorbed by the LAB and/or yeast biomass sample, B is the concentration of AFB_1 added to the buffer (1.0 µg/mL in buffer solution), C is the AFB_1 concentration in the buffer solution plus yeast and/or LAB inactivated cells after centrifugation, and D is the concentration of any interferences in the negative control (buffer solution+yeast/LAB cells).

Equação 1:

$$A = \frac{[B - (C - D)]}{B} \times 100 \tag{1}$$

Stability testing of the AFB_1 complex formed with all the above-mentioned treatments was carried out by the method described by Ismail et al. (2017). The pellets obtained after centrifugation of binding assays were exposed to multiple washings with PBS. The supernatant from each washing was analyzed for the amount of AFB_1 released.

2.3 Statistical analysis

The results were subjected to analysis of variance, in accordance with the general linear model (GLM) of SAS® (2004), to check for significant differences between means. When applicable, the Fisher's least significant difference (LSD) test was used for comparison between means (rejection level: $\alpha = 0.05$). Binding percentages ≥ 90% were considered as satisfactory ability to remove AFB_1 in solution.

3 RESULTS AND DISCUSSION

Our objective was to evaluate the in vitro ability of *S. cerevisiae* and *L. rhamnosus* to bind to AFB_1 in PBS solution, as well as to measure the stability of this binding.

Table 1 shows the AFB_1 at 1.0 μg/mL prepared in 0.1 M PBS at pH 3.0 and pH 6.5 after binding to *S. cerevisiae*, LAB, and *S. cerevisiae*/LAB. Lower AFB_1 levels were found in PBS after treatment with *S. cerevisiae*/LAB in combination, containing a biomass of 1×10^{10} inactivated cells, with values ranging from 31.5 ± 0.002 μg mL^{-1} in pH 3.0 and 27.9 ± 0.002 μg mL^{-1} in pH 6.5, representing an AFB_1 reduction of 61.9 and 68.1%, respectively.

The second-best response was achieved using the *S. cerevisiae* (Fermentis K-97, SafAle, Bruggeman, Belgium) 1.0 × 1010 yeast cells/g, with values ranging from 35.2 ± 0.002 μg mL^{-1} in pH 3.0 and 52.6 ± 0.002 μg mL^{-1} in pH 6.5, representing an AFB_1 reduction of 57.4 and 39.8%, respectively. The isolated use of *L. rhamnosus* HOWARU® LYO 40 DCU 1.0×10^{10} cells/g showed values ranging from 66.1 ± 0.002 μg mL^{-1} in pH 3.0 and 56.6 ± 0.002 μg mL^{-1} in pH 6.5, representing an AFB_1 reduction of 20.1 and 35.3%, respectively.

In an *in vitro* study conducted by Yiannikouris et al. (2021) on the adsorption capacity of a yeast cell wall (*S. cerevisiae*)-based adsorbent, the percentage of bound AFB_1 ranged from 81 to 94%, and the average percentage adsorbed was 89% when tested at pH 3.0, values higher than those found in our study.

A study using heat-inactivated *S. cerevisiae* and LAB strains, alone or in combination, showed that both *S. cerevisiae* cells, alone or when combined with LAB strains, showed the ability to reduce AFM_1 levels in milk, a fact that corroborates the results found in our work. The results obtained are in agreement with those reported by Zolfaghari et al. (2020), who reported that bacteria and yeast isolates showed an ability to reduce AFB_1. The binding capacity of *Lactobacillus sp*. ranged from 8.38 to 31.14%, and AFB_1 binding obtained by *S. cerevisiae* was 30.46%. El-Nezami et al. (1998; 2002) reported that strains of *L. rhamnosus* showed binding capacity with AFM_1 in whole milk varying between 36 and 63% (Luo et al., 2020).

Elsanhoty et al. (2014) and Pourmohammadi et al. (2022) reported that *L. rhamnosus* was efficient in binding with AFB_1. Hernandez-Mendoza et al. (2009) and Huang et al. (2017) found AFB_1 detoxification binding percentages by *L. rhamnosus* of 44.89% at pH 6.5. Zinedine et al. (2005) reported that *L. rhamnosus* achieved a rate of 44.89% AFB_1 removal at pH 6.5; these values are higher than those found in our study.

The differences between the results cited by other authors and the results obtained in our study can be explained by findings from El-Nezami et al. (1998), who demonstrated that the rate of AFB_1 removal is time-dependent, and by Kasmani and Mehri (2015), who reported that the AFB_1 removal capacity by *L. rhamnosus* is dependent on time, temperature, and pH.

Pourmohammadi et al. (2022) demonstrated that the combination of LAB strains increases the rate of adsorption of toxins when compared with the isolated use of the strains, a fact that would explain the better adsorption results obtained with the combination of *S. cerevisiae*/LAB in our study.

Table 2 shows the stability testing of the AFB_1 complex formed with all treatments at pH 3.0 and 6.5 after binding to *S. cerevisiae*, LAB, and *S. cerevisiae*/LAB. The stability of the mycotoxin microorganism complex is a measure of the efficiency of the strain as a decontaminating agent. After exposure to multiple washings with PBS, the supernatant from each washing was analyzed to determine the amount of AFB_1 released. At pH 3.0, isolated *S. cerevisiae* showed the highest percentage of AFB_1 retention, 85.7%, followed by the combination of LAB/*S. cerevisiae* with 78.1% and isolated LAB with 56.2%. At pH 6.5, the combination of LAB/*S. cerevisiae* showed the highest percentage of AFB_1 retention with 82.1%, followed by the isolated *S. cerevisiae* with 78.2%, and the isolated LAB with 71.8%.

There are few studies on the capacity of aflatoxin desorption by LAB strains. Serrano-Niño et al. (2013) reported that aflatoxin was released in smaller percentages than ours when using *L. rhamnosus*, and after the first wash with PBS, the percentage of aflatoxin released was 3.18% and after two washes, no aflatoxin was found in the wash solution.

El-Nezami et al. (2000) reported that the AFB_1/adsorbent complex incubated *in vivo* in the intestinal lumen for 60 min proved to be more stable than the complex incubated *in vitro* for the same period, where *L. rhamnosus* released between 13.9 and 64.2% of AFB_1. In our study, the percentage of in vitro release of AFB_1 using isolated *L. rhamnosus* was similar (28.2%).

Table 1. Aflatoxin B_1 at 1.0 μg/mL prepared in 0.1 M potassium phosphate buffer at pH 3.0 and pH 6.5 after binding to *Saccharomyces cerevisiae*, *L. rhamnosus*, and *L. rhamnosus* + *S. cerevisiae*.

Sample	AFB_1 remaining inPBS[b] (μg mL^{-1})		Reduction of AFB_1 (%)	
	pH 3.0	pH 6.5	pH 3.0	pH 6.5
PBS	82.7 ± 0.4^{aA}	87.5 ± 2.2^{aA}	–	–
SC	35.2 ± 0.4^{bA}	52.6 ± 3.0^{bB}	57.4 ± 0.7^{aA}	39.8 ± 1.9^{aB}
LAB	66.1 ± 4.6^{cA}	56.6 ± 1.1^{bB}	20.1 ± 5.5^{bA}	35.3 ± 1.2^{bB}
LAB/SC	31.5 ± 2.1^{dA}	27.9 ± 1.0^{cA}	61.9 ± 2.6^{cA}	68.1 ± 1.1^{cA}

AFB_1: aflatoxin B_1. PBS: AFB_1 at 1.0 μg/mL prepared in 0.1 M potassium phosphate buffer (PBS). SC: *S. cerevisiae* 1.0×10^{10} yeast cells/g. LAB: *L. rhamnosus* 1.0×10^{10} cells/g. LAB/SC: *L. rhamnosus* + *S. cerevisiae*. Both the *L. rhamnosus* strain in the lyophilized starter culture and *S. cerevisiae* cells were inactivated by autoclaving the material at 121°C for 10 min. Values expressed as mean ± standard deviation of samples analyzed in triplicate. [a–d]In the same column, means followed by different letters differ significantly ($p < .05$). [A–B]In the same line, means followed by different letters differ significantly ($p < .05$).

Table 2. Stability testing of aflatoxin B_1 complex formed with all treatments at pH 3.0 and 6.5 after binding to *Saccharomyces cerevisiae*, *L. rhamnosus*, and *Saccharomyces L. rhamnosus + S. cerevisiae*.

Sample	AFB_1 released in PBS[b] ($\mu g\ mL^{-1}$)		% of AFB_1 bound	
	pH 3.0	pH 6.5	pH 3.0	pH 6.5
SC	6.8 ± 0.8^{aA}	7.6 ± 2.0^{aA}	85.7 ± 1.6^{aA}	78.2 ± 5.9^{aA}
LAB	7.3 ± 1.9^{aA}	8.7 ± 3.6^{aA}	56.2 ± 11.7^{aA}	71.8 ± 11.6^{aA}
LAB/SC	11.2 ± 2.3^{bA}	10.6 ± 1.6^{bA}	78.1 ± 3.8^{bA}	82.1 ± 2.6^{bA}

For the aflatoxin retention assay, the pellet was then suspended in 1 mL of potassium phosphate buffer. This process was repeated five times. AFB1: aflatoxin B_1. SC: *S. cerevisiae* 1.0×10^{10} yeast cells/g. LAB: *L. rhamnosus* 1.0×10^{10} cells/g. LAB/SC: *L. rhamnosus*+*S. cerevisiae*. The percentage of AFB_1 bound to cells was calculated as the difference between the total AFB_1 and the amount of free AFB_1. Values expressed as mean ± standard deviation of samples analyzed in triplicate. [a–b]In the same column, means followed by different letters differ significantly ($p < .05$). [A–B]In the same line, means followed by different letters differ significantly ($p < .05$).

In the work by Bovo et al. (2013), the AFM_1 released by the LAB ranged from 40.57 to 87.37%. Elgerbi et al. (2006) reported the rate of AFM_1 released by LAB strains of 85.7% in the first wash and 100% after the third wash. On the other hand, Kabak and Var (2008) reported percentages of AFM_1 released between 5.62 and 8.54%. In our study, part of the toxin was released from the LAB/AFB_1 complex, and the stability rate of the adsorbent/aflatoxin complex ranged from 56.2 to 71.8% after washes, suggesting a weak non-covalent binding. These different results can be explained by differences in the binding sites between the different strains used, as well as by the formation of a cross-linked matrix formed by the binding between cell walls and aflatoxin molecules, a fact that reduces the rate of aflatoxin release.

Martínez et al. (2019) showed that *S. cerevisiae* desorbed all the adsorbed toxin (100%) and *L. rhamnosus* desorbed 86%. These findings differ from our study, which demonstrated a greater capacity for stability of the mycotoxin/adsorbent complex, for both *S. cerevisiae* and *L. rhamnosus*.

Comparison of the results obtained using the combination of *S. cerevisiae* with LAB with other studies is difficult because there are no previous data on the removal of aflatoxins by this association. However, in our work, it was observed that the association of *S. cerevisae* with LAB resulted in better adsorption rates and higher stability rates of the mycotoxin complex.

4 CONCLUSIONS

The best AFB_1 adsorption rate occurred with the use of LAB/*S. cerevisae* at both pH 3.0 (61.9%) and 6.5 (68.1%), followed by *S. cerevisiae* isolated at pH 3.0 (57.4%). Regarding the stability of the adsorbent/mycotoxin complex, they showed greater stability to *S. cerevisae* isolated at pH 3.0 (85.7%) and less stability to LAB at pH 3.0 (56.2%). The three methods were effective in removing AFB_1 and in the stability of the adsorbent/mycotoxin complex.

The study may be useful in selecting the most efficient microorganisms to remove AFB_1. Although the tests were performed in vitro, the results suggest that the addition of LAB or *S. cerevisiae* to food would help prevent aflatoxicosis. Additional studies are needed to assess the impact on production costs of each adsorbent, in order to assess the feasibility of commercial application in the food industry.

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