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# Evaluation of *Saccharomyces cerevisiae* strains as probiotic agent with aflatoxin $B_1$ adsorption ability for use in poultry feedstuffs

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In this study the aflatoxin $B_1$ ($AFB_1$) removal capacity, the tolerance to salivary and gastrointestinal conditions, autoaggregation and coaggregation with pathogenic bacteria of *Saccharomyces cerevisiae* strains isolated from broiler feces, were evaluated. Only four of twelve isolated strains were identified as *Saccharomyces cerevisiae* using molecular techniques. The results obtained in $AFB_1$ binding studies indicated that the amount of $AFB_1$ removed was both strain and mycotoxin-concentration dependent. Therefore, a theoretical model was applied in order to select the most efficient strain to remove $AFB_1$ in a wide range of mycotoxin concentration. The results indicated that *S. cerevisiae* 08 and *S. cerevisiae* 01 strains were the most efficient microorganisms in the mycotoxin removal. Viability on simulated salivary and gastrointestinal conditions was investigated and *S. cerevisiae* 08 strain showed the best results, achieving 98% of total survival whereas *S. cerevisiae* 01 reached only 75%. Autoaggregation and coaggregation assays showed *S. cerevisiae* 08 as the most appropriate strain, mainly because it was the unique strain able to coaggregate with the four bacterial pathogens assayed. Consequently, *S. cerevisiae* 08 is the best candidate for future *in vivo* studies useful to prevent aflatoxicosis. Further quantitative *in vitro* and *in vivo* studies are required to evaluate the real impact of yeast-binding activity on the bioavailability of $AFB_1$ in poultry. However, this study could be useful in selecting efficient strains in terms of $AFB_1$ binding and provide an important contribution to research into microorganisms with potential probiotic effects on the host.

**Keywords:** *Saccharomyces cerevisiae*, aflatoxin $B_1$, binding efficiency, gastrointestinal conditions, adhesion abilities.

## Introduction

Feeds contaminated with mycotoxins have a health risk to animals and, as a consequence, may cause big economical losses due to the lower efficacy of animal husbandry. In addition, directly or indirectly (animal by-products) contaminated foods may also constitute health risk to humans.[1] Aflatoxins (AFs) are the most important mycotoxins produced by *Aspergillus flavus, A. parasiticus* and *A. nomius*.[2-4] Among the various dangerous AFs and their metabolites, aflatoxin $B_1$ ($AFB_1$) is the most widespread and potent naturally occurring hepatocarcinogen in animals and humans, and the diet is the best-known route of exposure.[5-7]

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Poultry is exposed to AFs by eating feeds prepared with contaminated raw materials and the produced mycotoxicosis, aflatoxicosis, apart from affecting their health reduces poultry production and affects the availability of certain products and their marketing.[8] Aflatoxicosis in poultry causes listlessness, anorexia with low growth rate, poor food utilization, weight gain decrease, egg production decrease, susceptibility increase to environmental and microbial stresses, and mortality.[9,10] Anemia, mutagenicity, carcinogenicity and teratogenicity were also reported.[11] The reduction of immune function which increases susceptibility to pathogenic bacteria, such as *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus* and *Enterobacter* spp. among others, is also associated with aflatoxicosis.[12,13]

Physical and chemical treatments have been used for feedstuff AFs detoxification. [14] However, $AFB_1$ is almost an unavoidable contaminant in the poultry industry. Thus, novel approaches have focused on preventing $AFB_1$ absorption in the gastrointestinal (GI) tract of humans and animals.[15-17] *Saccharomyces cerevisiae* and lactic acid bacteria (LAB) have been used in functional feeds as probiotics

and as potential mycotoxin decontaminating microorganisms by their ability to bind $AFB_1$.[18,19] Inclusion of such microbes in the diet may reduce the toxic effects of mycotoxins on animals since an $AFB_1$–microorganism complex may decrease availability of the mycotoxin and consequently its absorption in the GI. Aflatoxin binding appears to be an adsorption phenomenon to cell wall components of LAB and yeast, and both the quantity and the stability of this complex are very important for selecting the most efficient microorganism to remove $AFB_1$.[20] Evidence from poultry feeding experiments have shown that the yeast cell wall-AFs complex can efficiently pass through the gut, resulting in protection from AFs induced toxicities.[21]

Microorganisms must be first subjected to expensive, complicated and long-lasting *in vivo* studies to be included in the poultry diet destined to prevent aflatoxicosis. Therefore, the selection of the most suitable microorganism to carry out these studies is very important thus the efficiency to remove $AFB_1$, the GI condition tolerance and potential antimicrobial properties are essential. Nevertheless, several publications reported microorganisms, mainly bacteria, to exert beneficial effects on the host,[13,22-24] and other authors studied *in vitro* bacteria and yeast with $AFB_1$ removal abilities separately.[19,25-29] In addition, Bueno et al.[20] developed a theoretical model able to quantify the efficiency of microorganisms in the $AFB_1$ detoxification by determining two parameters, the process equilibrium constant ($K_{eq}$) and the number of $AFB_1$ binding sites per microorganism (M). To our knowledge only Armando et al.[30] showed both potential beneficial properties and $AFB_1$ decontamination abilities from *S. cerevisiae* strains, in the same report. To understand their binding mechanism and beneficial properties, it becomes necessary to allow the optimization and safe dietary application of this methodology.

In the present study, the removal of $AFB_1$ and potential beneficial properties, were investigated. The objective of this research was to perform an *in vitro* evaluation of the ability of *S. cerevisiae* strains isolated from broiler feces, to remove $AFB_1$ from liquid media. In addition, some potential beneficial properties such as salivary and GI condition tolerance, autoaggregation and coaggregation with pathogenic bacteria were evaluated.

## Materials and methods

### *Yeast strains isolation and characterization*

Yeast strains were isolated from six fresh broiler feces samples randomly collected in three poultry farms (two samples per farm) from Rio Cuarto region, Argentina. Ten grams of fresh feces samples were added to 90 mL peptone water (0.1% wt $v^{-1}$) and shaken at room temperature for 30 min. Then, decimal serial dilutions were carried out and 100 $\mu$L of each dilution were inoculated in duplicate in Yeast Extract Peptone Dextrose (YPD) agar (5 g of yeast extract, 5 g of peptone, 40 g of dextrose and 20 g of agar, in 1 liter of deionized water). Plates were incubated aerobically at 25°C for 48 h. The colonies taken randomly from the total plates were analyzed morphologically and twelve of them were identified as yeasts and after their purification in YPD agar they were maintained as frozen stocks at –20°C in presence of 30% (v $v^{-1}$) glycerol as a cryoprotective agent. These yeast strains were examined for their phenotypic characteristics according to Pitt and Hocking.[31] Species designation was confirmed by molecular techniques.

### *Molecular identification of yeast strains*

The yeast strains isolated from broiler feces were sent to Instituto Nacional de Tecnología Agropecuaria (INTA), Lujan de Cuyo, Mendoza, Argentina. Identification was obtained through the analysis of rRNA,[32] and for the identification of yeast at species level a map restriction was done.[33-35]

From the total of yeast strains isolated, four of them belonged to *Saccharomyces cerevisiae* species. As they are considered GRAS (Generally Regarded As Safe) microorganisms, further studies with only these strains were carried out. They were identified as: *S. cerevisiae* 01, *S. cerevisiae* 03, *S. cerevisiae* 05 and *S. cerevisiae* 08.

### *Microorganisms and culture conditions*

Four bacterial pathogens, *Staphylococcus aureus, Escherichia coli, Enterobacter cloacae* and *Salmonella enterica* ssp. *enterica*, from clinical isolates, were grown on Nutrient Broth (Sigma-Aldrich, St. Louis, MO, USA) under aerobic conditions for 12 h at 37°C, using inocula at 0.3 % (v $v^{-1}$) from an overnight culture at 37°C.

*Saccharomyces cerevisiae* RC016 was obtained from the culture collection of National University of Río Cuarto, Córdoba, Argentina.

All yeast strains were grown on YPD broth (5 g of yeast extract, 5 g of peptone, 40 g of dextrose, in 1 liter of deionized water) in a orbital incubator at 150 rpm for 24 h at 25°C. The inocula of the yeast strains were prepared from an overnight culture in YPD broth at 25°C. Then, 250 mL Erlenmeyer flasks containing 100 mL of YPD were inoculated with 1 mL of the respective inocula. Cell suspension concentration was determined using a haemocytometer. Viability was confirmed by standard count methods using MRS or YPD agar.

### *Aflatoxin $B_1$ binding assay*

The $AFB_1$ binding assay was performed according to Bueno et al.[20] with modifications. Briefly, a stock solution of solid $AFB_1$ (Sigma-Aldrich, St. Louis, MO, USA) was suspended in benzene-acetonitrile (97:3 v $v^{-1}$) to obtain an $AFB_1$ concentration of 1 mg/mL. The benzene-acetonitrile

was evaporated by heating at 70°C for 10 min, and 50 $\mu$L of methanol was added and brought to final volume with phosphate-buffered saline (PBS) (0,022 M), pH 7.3.

Cells were washed twice with PBS and incubated at 37°C for 30 min in a shaking bath with 1 ml of PBS containing $AFB_1$ (0.3; 0.625; 0.9; 1.25; 2.0 and 2.5 $\mu$g/mL). Then, cells were pelleted by centrifugation at 5000 g for 15 min at room temperature, and the supernatant containing unbound $AFB_1$ was collected and stored at −20°C until HPLC analysis. Positive (PBS + mycotoxin) and negative (PBS + cells) controls were included for all experiments.

### *Quantification of aflatoxin $B_1$ by HPLC*

Aflatoxin $B_1$ analysis was performed by HPLC according to the methodology proposed by Trucksess et al.[36] Aflatoxin $B_1$ was quantified by reversed-phase HPLC with Hewlett Packard series 1100 HPLC equipment, with fluorescence detection (Hewlett Packard 1046 A): λ exc 360 nm; λ em 440 nm and a chromatographic column: C18 column (Supelcosil LC-ABZ, Supelco; 150 × 4.6 mm, 5 $\mu$m particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20 × 4.6 mm, 5 $\mu$m particle size). Methanol-acetonitrile-water (1:1:4 v $v^{-1}$) was used as the mobile phase at a flow rate of 1.5 mL/min. The retention time was around 5 min. Standard curves were prepared with different levels of $AFB_1$ (Sigma) in PBS (pH 7.3). The toxin was quantified by correlating peak areas of samples with those of the standard curves. The detection limit of the technique was 1 ng/g. Samples of the supernatants and standard of $AFB_1$ were derivatized prior to injection, an aliquot of 200 $\mu$L of the sample was derivatized with 700 $\mu$L trifluoroacetic acid-acetic acid-water (20:10:70 v $v^{-1}$).

### *Efficiency parameters*

To further study the interaction between the yeast strains and the mycotoxin, we applied the model developed by Bueno et al.[20], which was proposed to explain the process of $AFB_1$ adsorption by LAB and yeast strains. Briefly, this model considers the attachment of $AFB_1$ molecules to the microorganism surface. The relationship between the amounts of the $AFB_1$ at the microorganism surface as a function of its solution concentration is described by an adsorption isotherm, which is linear at the beginning then transitioning to a plateau. This type of isotherm can be described by Equation 1:

$$\text{Adsorption} = M\,[\text{toxin}]_{\times} K_{eq}/1 + [\text{toxin}]\,K_{eq} \quad (1)$$

Where M is the maximum number of adsorption sites per microorganism, and $K_{eq}$ (expressed in litres per mole) is equivalent to the affinity of toxin molecules for the adsorption sites. The linearized form of the isotherm is the double-reciprocal plot from the saturation curve, and from the slope and interception of the resulting line, factors M and $K_{eq}$ can be determined. The most efficient microorganism would be the one which has maximal M and $K_{eq}$ values or simply the higher product of them ($M \times K_{eq}$).

### *Determination of survival after simulated passage through salivary and gastrointestinal condition*

Salivary and GI *in vitro* tolerance was tested as described by Fernández et al.[37] The composition of the used solutions was closest to the real composition of salivary, gastric and intestinal juice from poultry.[38] Briefly, 25 mL of YPD broth were inoculated as it was described above and incubated with agitation at 25°C for 24 h. An aliquot (4 mL) was washed in sterile saline solution (NaCl 0.9%) and centrifuged at 5000 g for 10 min, the pellets were resuspended in 4 mL of artificial salivary secretion with the following composition: α amylase (α amylase from *Bacillus subtilis,* Sigma-Aldrich, St. Louis, MO, USA) 2 mg/mL and lysozyme (lysozyme chloride form from chicken egg white, Sigma-Aldrich, St. Louis, MO, USA) 2 mg/mL in saline solution pH 6.5 and incubated at 37°C for 15 min. Aliquots (50 $\mu$L) were taken for the enumeration of viable cells at 0 and 15 min. To assay the gastric condition tolerance, 8 mL of simulated gastric juice [NaCl (0.125 M); KCl (0.007 M); $NaHCO_3$ (0.045 M) and pepsin (pepsin from porcine gastric mucosa, Sigma-Aldrich, St. Louis, MO, USA) 3 g/L; final pH 3 was adjusted with HCl] were added to the 4 mL of artificial salivary secretion. Then, cells were incubated at 37°C for 60 min under agitation (200 rpm) to simulate the peristalsis. After incubation, aliquots (50 $\mu$L) were taken for cell enumeration. The effect of intestinal conditions was determined after centrifugation and pellets were suspended in 12 mL of artificial intestinal fluid: trypsin (trypsin from bovine pancreas, Sigma-Aldrich, St. Louis, MO, USA) 1 mg/mL; chymiotrypsin (chymiotrypsin from bovine pancreas, Sigma-Aldrich, St. Louis, MO, USA) 1 mg/mL; ox-gall bile salts (Sigma-Aldrich, St. Louis, MO, USA) 0.3 % (wt $v^{-1}$) in water and adjusted to pH 8 with NaOH. The cellular suspensions were incubated at 37°C for 120 min under agitation (200 rpm) and samples (50 $\mu$L) for viable cells counts were taken.

### *Aggregation assays*

Specific cell-cell interactions were determined using autoaggregation and coaggregation assays.[39] The yeast cells were harvested at 5000 g for 10 min at room temperature, washed with PBS and resuspended in PBS to approximately 0.7 Optical Density (OD) units at 600 nm. For the autoaggregation assay, each yeast suspension (2 mL) was vortexed for 10 s and its OD was determined ($T_0$), and then incubated at 37°C for 2 h without agitation. The absorbance of the upper suspension was measured at 600 nm ($T_1$) and the autoaggregation was calculated according to the Equation 2:

$$\text{Autoaggregation}\,\% = [1 - (O.D._{600}\ T_1\ /\ T_0)] \times 100. \quad (2)$$

The method for preparing the cell suspensions for coaggregation was the same as that used for autoaggregation assay, both yeast and bacteria, were washed with PBS and suspended in PBS to approximately 0.7 O.D. Equal volumes (2 mL) of yeast strain and pathogenic bacteria (*Staphylococcus aureus, Escherichia coli, Enterobacter cloacae and Salmonella enterica* ssp. *enterica*) were mixed, vortexed for 10 s and incubated at 37°C during 2 h without agitation. Then, the absorbance of the upper suspension of the mixture described above (mix) and the yeast and pathogen suspensions alone were measured at 600 nm. The coaggregation was calculated according to the Equation 3:

$$\text{Coaggregation}\,\% = [1 - \text{O.D.}_{600\ \text{mix}}/(\text{O.D.}_{600\ \text{yeast}} + \text{O.D.}_{600\ \text{pathogen}})/2] \times 100 \quad (3)$$

### *Statistical analysis*

All the studies were done by three experiments in duplicate. Data were analyzed by analysis of variance (ANOVA). Means were compared using the Fisher's protected Least Significant Difference test (LSD test). The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC). The results are considered statistically different only at $P < 0.05$.

## Results

### *Ability of* S. cerevisiae *strains to bind aflatoxin $B_1$*

Table 1 shows the ability to remove $AFB_1$ from contaminated liquid medium by *S. cerevisiae* strains isolated from broiler feces and *S. cerevisiae* RC 016 isolated from pig gut by Armando et al.,[30] and confronts their results with ours on yeast strains from another source. When the microorganisms were assayed at three different $AFB_1$ concentrations (50; 100 and 500 ng/mL), the amount of AFs bound was mycotoxin-concentration dependent and the level of binding appeared to vary between the strains indicating the microorganism-specific nature of binding. Thus, when $AFB_1$ concentration was 50 ng/mL, *S. cerevisiae* RC 016 was the most effective strain, but *S. cerevisiae* 08 was the best when $AFB_1$ concentration changed to 100 ng/mL and with 500 ng/mL of $AFB_1$ *S. cerevisiae* RC 016 was again the most efficient strain. In addition, *S. cerevisiae* 01 and *S. cerevisiae* 03 removed $AFB_1$ with similar ability when the $AFB_1$ concentrations were 50 and 100 ng/mL, but *S. cerevisiae* 01 was more effective at 500 ng/mL. Adsorption isotherms of $AFB_1$ by *S. cerevisiae* strains are shown in Figure 1. The shape of the isotherm shows linearity at low values of $AFB_1$ whereas at high concentrations it shows a transition to a plateau (Fig. 1 A). The linearized form of the isotherm is the double-reciprocal plot from the saturation curve (Equation 4: $1/\text{adsorption} = 1/[AFB_1]\ 1/M\ K_{eq} + 1/M$), is shown in Figure 1 B and allows to determine $K_{eq}$ and M values from each *S. cerevisiae* strain (equal for *S. cerevisiae* RC 016, data not shown).

The results shown in Table 2 indicated that the most efficient microorganisms in $AFB_1$ removal were *S. cerevisiae* 01 and *S. cerevisiae* 08, mainly due to their high values of M, whereas the $K_{eq}$ values were of similar order for the five studied strains.

### *Resistance through simulated salivary and gastrointestinal passage*

*Saccharomyces cerevisiae* strains were tested to determine the effects of exposure to simulated salivary and GI conditions and the results are shown in Table 3. All the yeast strains tested, with the exception of *S. cerevisiae* 01 (89% survival) showed an optimal survival percentage (100%) when exposed to salivary conditions. Instead, when *S. cerevisiae* strains were exposed to an artificial gastric juice during 1 h, the strains displayed a different behavior and

**Table 1.** Aflatoxin $B_1$ removal by *Saccharomyces cerevisiae* strains at three different mycotoxin concentrations.

| | *$AFB_1$ concentration (ng $mL^{-1}$)* | | | | | |
|---|---|---|---|---|---|---|
| | *$AFB_1$ binding$^+$* | | | | | |
| | *50* | | *100* | | *500* | |
| *Strains** | *(ng/mL)* | *%* | *(ng/mL)* | *%* | *(ng/mL)* | *%* |
| *S. cerevisiae* RC 016 | **41.6 ± 1.9$^a$** | **82.0** | 49.1 ± 1.4$^b$ | 48.7 | **328.8 ± 5.2$^a$** | **65.5** |
| *S. cerevisiae* 01 | 19.3 ± 1.2$^{bc}$ | 38.6 | 31.7 ± 1.2$^c$ | 31.7 | 164.0 ± 6.9$^c$ | 32.8 |
| *S. cerevisiae* 03 | 23.3 ± 2.9$^b$ | 46.6 | 34.5 ± 1.8$^c$ | 34.5 | 128.7 ± 5.8$^d$ | 25.7 |
| *S. cerevisiae* 05 | 16.7 ± 1.2$^c$ | 33.4 | 24.0 ± 1.7$^d$ | 24.0 | 92.0 ± 6.9$^e$ | 18.4 |
| *S. cerevisiae* 08 | 23.2 ± 2.8$^b$ | 46.4 | **58.9 ± 0.1$^a$** | **58.9** | 187.0 ± 7.2$^b$ | 37.4 |

*$10^7$ total cells/mL.

$^+AFB_1$ binding was calculated as the difference between the $AFB_1$ in the medium and the amount of free $AFB_1$ (supernatant).

Data are means ± standard deviations from three experiments in duplicate. Means within the same column with different letters are significantly different ($P < 0.05$).

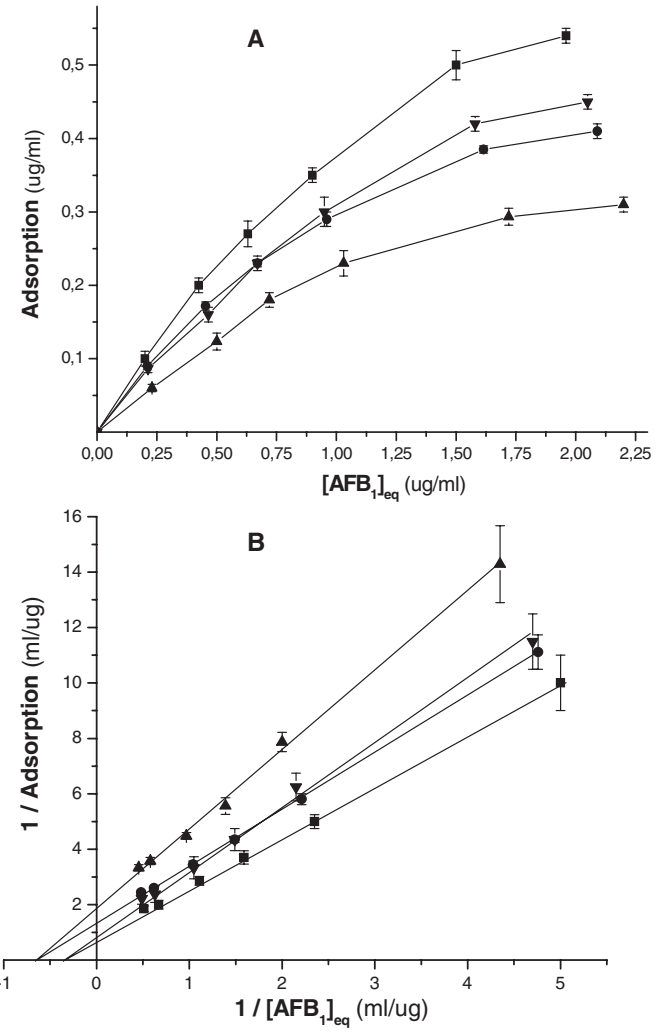


**Fig. 1.** Adsorption isotherms of aflatoxin $B_1$ by *Saccharomyces cerevisiae* strains. Aliquots of 1 mL of total cells [$3.4 \times 10^7$ cells for *S. cerevisiae* 01 (■), $5.4 \times 10^7$ cells of *S. cerevisiae* 03 (●), $4.9 \times 10^7$ cells for *S. cerevisiae* 05 (▲) and $2.7 \times 10^7$ cells for *S. cerevisiae* 08 (▼)] were suspended in PBS in the presence of 0.3; 0.625; 0.9; 1.25; 2.0 and 2.5 $\mu$g/mL of $AFB_1$. Mycotoxin bound to cells was calculated as the difference between the total $AFB_1$ and the amount of free $AFB_1$ ($[AFB_1]_{eq}$). (A) Saturation curve. (B) Inverse plot of the same data as (A). Data are means ± standard deviations from three experiments for duplicate.

only *S. cerevisiae* 08 was not affected under an acidic environment. Regarding the simulated intestinal condition all *S. cerevisiae* strains tested were resistant to this condition for 2 h.

In summary, *S. cerevisiae* 08 showing the minor percentage of total decrease in viability (2%, $P < 0.05$) was the most resistant strain to the salivary and GI conditions, while the other strains reported a total decrease in viability from 9% to 25% (Table 3) during the three treatments.

### *Autoaggregation and coaggregation abilities*

Three autoaggregation phenotypes are described according to their autoaggregation percentage, and those exceeding 80% are interpreted as a strong autoaggregation. [22] Table 4 shows this property for the four *S. cerevisiae* strains assayed. Except *S. cerevisiae* 05, the other strains showed strong autoaggregation and within the same order (85%, $P < 0.05$).

Coaggregation between *S. cerevisiae* strains and the selected pathogenic bacteria are shown in Table 5. All tested yeast strains were able to coaggregate efficiently with *Staphylococcus aureus* (≥ 25%). The assays between the other pathogenic bacteria strains and *S. cerevisiae* 01, *S. cerevisiae* 03 and *S. cerevisiae* 05 showed a poor coaggregation (≤ 10%); whereas *S. cerevisiae* 08 strain always reached the higher values (15–26%). Therefore, the unique yeast strain able to coaggregate efficiently with all the pathogen bacterial strains tested was *S. cerevisiae* 08.

## Discussion

Considering the impact of mycotoxins in poultry production, the application of different methods to reduce the severity and/or consequences of mycotoxicosis is desirable. Several prevention and control strategies have been applied in order to reduce mycotoxin levels in food intended to animal consumption. Most of the approaches have not been adopted due to high costs, practical difficulties involved in the detoxification process and/or by the impossibility to guarantee the results. Therefore, the hypothesis that foods and feeds can always be potentially contaminated with AFs

**Table 2.** Total binding sites per microorganism (M), equilibrium constant ($K_{eq}$) and efficiency ($M \times K_{eq}$) for *Saccharomyces cerevisiae* strains.

| *Strains* | *M ($1\times10^7$ sites/cell)* | *$K_{eq}$ ($1\times10^5$ $M^{-1}$)* | *Efficiency ($1\times10^{12}$)* |
|---|---|---|---|
| *S.cerevisiae* RC 016 | $3.30 \pm 0.21^{b}$ | $1.20 \pm 0.10^{b}$ | $3.96 \pm 0.29^{c}$ |
| *S. cerevisiae* 01 | $7.47 \pm 0.57^{a}$ | $1.34 \pm 0.11^{b}$ | $10.01 \pm 0.30^{a}$ |
| *S. cerevisiae* 03 | $2.59 \pm 0.20^{c}$ | $2.13 \pm 0.19^{a}$ | $5.52 \pm 0.13^{b}$ |
| *S. cerevisiae* 05 | $2.04 \pm 0.14^{d}$ | $1.96 \pm 0.18^{a}$ | $4.00 \pm 0.38^{c}$ |
| *S. cerevisiae* 08 | $7.66 \pm 0.54^{a}$ | $1.34 \pm 0.15^{b}$ | $10.26 \pm 0.46^{a}$ |

Data are means ± standard deviations from three experiments in duplicate. Means within the same column with different letters are significantly different ($P < 0.05$).

**Table 3.** Determination of survival of *Saccharomyces cerevisiae* strains during salivary, gastric and intestinal simulated conditions.

| S. cerevisiae *strains* | *Log (CFU/mL) Prior to assay* | *Log (CFU/mL) After 15 min in artificial salivary secretion* | *Survival* (%)* | *Log (CFU/mL) After 60 min in artificial gastric conditions* | *Survival (%)* | *Log (CFU/mL) After 120 min in artificial intestinal conditions* | *Survival (%)* | *Total decrease in viability** (%)* |
|---|---|---|---|---|---|---|---|---|
| 01 | $5.51 \pm 0.01$ | $5.46 \pm 0.01$ | $88.9 \pm 1.9^{b}$ | $5.40 \pm 0.01$ | $87.0 \pm 1.9^{bc}$ | $5.39 \pm 0.01$ | $97.9 \pm 1.5^{a}$ | $24.6 \pm 2.1^{a}$ |
| 03 | $5.63 \pm 0.01$ | $5.63 \pm 0.02$ | $100.0 \pm 2.0^{a}$ | $5.59 \pm 0.01$ | $91.0 \pm 2.1^{b}$ | $5.59 \pm 0.02$ | $100.0 \pm 2.0^{a}$ | $8.9 \pm 2.0^{c}$ |
| 05 | $5.80 \pm 0.01$ | $5.80 \pm 0.01$ | $100.0 \pm 1.0^{a}$ | $5.73 \pm 0.01$ | $84.6 \pm 1.9^{c}$ | $5.73 \pm 0.02$ | $100.0 \pm 2.0^{a}$ | $15.4 \pm 2.2^{b}$ |
| 08 | $5.90 \pm 0.02$ | $5.89 \pm 0.01$ | $97.5 \pm 2.1^{a}$ | $5.89 \pm 0.02$ | $100.0 \pm 2.0^{a}$ | $5.89 \pm 0.01$ | $100.0 \pm 2.0^{a}$ | $2.0 \pm 1.8^{d}$ |

Data are means ± standard deviations from three experiments in duplicate. Means within the same column with different letters are significantly different ($P < 0.05$).
Survival (%) and the total decrease in viability (%) were determined according Equation 5 and 6, respectively.
*Survival % = [(CFU $mL^{-1}$ final)/(CFU $mL^{-1}$ initial)] × 100 (5)
**Total decrease in viability % = [(CFU/mL initial – CFU/mL final)/CFU/mL initial] × 100. (6)



should be considered. Then, a promising alternative is the use of microorganisms with the ability to remove mycotoxins and at the same time with some potential beneficial properties on the host. Inclusion of such microbes in the diet may reduce the toxic effects of $AFB_1$ on the animal organism, since the $AFB_1$-microorganism complex may decrease mycotoxin availability and consequently its absorption in the gastrointestinal tract.[16]

In the poultry industry, *S. cerevisiae* has been used as general performance promoter in poultry feeds and has recently been shown to have beneficial effects against $AFB_1$ exposure.[40]

In this study twelve yeast strains were isolated from broiler feces, four of them were characterized as *Saccharomyces cerevisiae* strains by molecular techniques and as they are considered GRAS microorganisms, further studies were carried out only with these strains. The strain origin and its characterization is important in the selection of any microorganism that will be included in animal diet, as this will indicate the presumed safety of the strain, enable postmarketing surveillance and meet requirements of legislative bodies and governmental control organs for exact information on the strain nomenclature.[41]

The evaluation of *S. cerevisiae* in this research is based on *in vitro* studies for the characterization of three abilities of the yeast strains: (*i*) the capacity to bind $AFB_1$, (*ii*) salivary and GI conditions tolerance and (*iii*) potential beneficial properties on the host.

In order to investigate the $AFB_1$-binding capacity of *S. cerevisiae* strains, the removal at three different mycotoxin concentrations was evaluated. Efficiency varied with the $AFB_1$ concentration employed, therefore it was not possible to determine the most efficient strain. Many researchers reported the most efficient microorganism in the removal of $AFB_1$ using a unique mycotoxin concentration.[26,27,29,40]

However, the result is only valid for the toxin concentration employed. Our results reveal that the selection of the most efficient microorganism in $AFB_1$ removal, based on a unique concentration of $AFB_1$, could lead to an erroneous conclusion because its efficiency changes when the mycotoxin concentration is modified. For that reason, the theoretical model proposed by Bueno et al.[20] was applied as a good tool to select the most efficient microorganism in the removal of $AFB_1$ in a wide range of mycotoxin concentration, since previous studies have shown that poultry feed $AFB_1$ contamination is variable.[42-45] Thus, the M and $K_{eq}$ parameters were useful to determine the $AFB_1$-binding efficiency of each assayed microorganism. The $K_{eq}$ values describe the interacting force between the mycotoxin and microorganism cell wall, indicating that the strains with the higher values of $K_{eq}$ will release fewer toxins during passage through the intestinal tract, precisely where AFs are efficiently absorbed, probably by passive diffusion.[16] The stability of the microorganism-mycotoxin complex in the GI tract is essential for the practical application of microorganisms as decontaminating agents.[46] Although our results shown statistically differences among $K_{eq}$ values of *S. cerevisiae* strains tested, these differences were

**Table 4.** Autoaggregation ability of *Saccharomyces cerevisiae* strains.

| S. cerevisiae *strains* | $OD_{600}$ $(t_0)$ | $OD_{600}$ $(t_1)$ | *Autoaggregation (%)* |
|---|---|---|---|
| 01 | $0.619 \pm 0.012$ | $0.095 \pm 0.014$ | $84.7 \pm 1.2^{a}$ |
| 03 | $0.807 \pm 0.018$ | $0.132 \pm 0.011$ | $83.6 \pm 2.3^{a}$ |
| 05 | $0.686 \pm 0.016$ | $0.217 \pm 0.010$ | $68.4 \pm 0.9^{b}$ |
| 08 | $0.755 \pm 0.017$ | $0.119 \pm 0.012$ | $84.3 \pm 1.6^{a}$ |

Absorbance of the upper suspension measured at 600 nm at 0 h **($t_0$)**. Absorbance of the upper suspension measured at 600 nm after incubation at 37°C for 2 h **($t_1$)**.
Data are means ± standard deviations from three experiments in duplicate. Values corresponding to the same letter are not significantly different within the same column according to Fisher's protected LSD test ($P < 0.05$).

**Table 5.** Coaggregation assay of *Saccharomyces cerevisiae* strains and pathogenic bacteria.

| | *Coaggregation (%)* | | | |
|---|---|---|---|---|
| *S. cerevisiae strains* | *Staphylococcus aureus* | *Escherichia coli* | *Enterobacter cloacae* | *Salmonella enterica ssp. enteric* |
| 01 | $35.4 \pm 2.1^{a}$ | $1.2 \pm 0.5^{c}$ | $2.0 \pm 1.1^{c}$ | $2.1 \pm 1.4^{c}$ |
| 03 | $36.0 \pm 2.5^{a}$ | $9.7 \pm 1.1^{b}$ | $9.3 \pm 0.5^{b}$ | $1.0 \pm 0.8^{c}$ |
| 05 | $29.3 \pm 2.0^{b}$ | $8.3 \pm 0.9^{b}$ | $1.0 \pm 0.6^{c}$ | $9.5 \pm 1.1^{b}$ |
| 08 | $25.3 \pm 1.7^{b}$ | $14.9 \pm 1.1^{a}$ | $18.4 \pm 2.1^{a}$ | $26.7 \pm 2.1^{a}$ |

Data are means ± standard deviations from three experiments in duplicate. Values corresponding to the same letter are not significantly different within the same column according to Fisher's protected LSD test ($P < 0.05$).



not enough to cause changes in the release of $AFB_1$ by PBS washings (data not shown). On the other hand, the differences in M values were higher than those obtained in the $K_{eq}$ values; consequently the $AFB_1$ binding efficiencies of *S. cerevisiae* 01 and *S. cerevisiae* 08 were from 2 to 2.5 times higher than the other evaluated yeast strains, demonstrating microorganism specificity in the removal process.

The main criterion for selecting a beneficial strain is the assessment of its tolerance to GI conditions, since the acid in the stomach and bile salts in the gut are the first biological barriers to be overcome after ingestion.[47] This property is very important because it enhances the yeast possibility to colonize the animal GI tract and thus manifests its potential beneficial properties. To our knowledge, while some authors studied exposure of yeasts to bile salts or low pH, separately, others did it without any addition of enzymes.[48,49] In this work, an improved *in vitro* methodology simulating the real poultry composition of salivary, gastric and small intestinal juice,[38] was employed for the selection of *S. cerevisiae* strains. Although three of the four *S. cerevisiae* strains under investigation demonstrated variable ability to tolerate salivary and gastric conditions. It is interesting to note that *S. cerevisiae* 08 was the best of all the conditions assayed, showing only 2% of total decrease in its viability ($P < 0.05$). These results indicated that a significant number of *S. cerevisiae* 08 cells could survive the salivary and GI transit, increasing their possibility to colonize the poultry gut. Similar results were reported by Van der Aa Kühle et al.[49] with *S. cerevisiae* and *S. cerevisiae* var. *boulardii* strains isolated from foods or beverages. Moreover, high withstand to intestinal transit, but variable tolerance to acidic conditions of *S. cerevisiae* strains isolated from infant feces and feta cheese was observed.[48] On the contrary, Pennacchia et al.[24] revealed several differences in the resistance capability among the tested yeast strains, since all of them demonstrated a high ability to tolerate exposure to low pH but 12 out of 22 yeast strains demonstrated a low survivability, because they did not tolerate the small intestinal transit (bile salts and pancreatin).

On the other hand, adhesion to intestinal cells is an important pre-requisite for colonization of probiotic strains and is a complex and multistep process involving contact of the probiotic cell membrane and the interacting surfaces.[50] *In vivo* evaluation of adhesion ability of microorganisms to intestinal cells is not easy to perform and is also expensive in terms of materials. However, several researchers have reported good relation between high autoaggregation percentage (formation of multicellular clumps among organisms of the same strain) and cell adhesion ability.[22,51,52] In addition the autoaggregation assays are an easy and reproducible tool for evaluating the adhesion to intestinal epithelial cells.[53] Moreover, as adhesion is also necessary for the initiation of host-pathogen interactions, the prevention of pathogenic bacteria adhesion to the epithelial cells of GI tract is an effective strategy for reducing the risk of foodborne illness.[39] Thus, coaggregation abilities may form a barrier that prevents colonization by pathogenic microorganisms which could constitute an important host defense mechanism against infection.[53]

For these reasons, in order to determine the potential beneficial properties of *S. cerevisiae* strains analyzed in this report, we evaluated their autoaggregation and coaggregation capacities as an approximation to their intestinal cell adhesion ability and prevention against bacterial pathogens colonization, respectively. The autoaggregation assays showed that only *S. cerevisiae* 05 should be discarded, as its autoaggregation percentage was below 80%. According to coaggregation ability, all *S. cerevisiae* strains studied coaggregated with *Staphylococcus aureus*, but only *S. cerevisiae* 08 coaggregated with all the tested pathogens and moreover, it showed the greater coaggregation ability with *Salmonella enterica* ssp. *enterica*, one of the most important pathogens in the poultry industry.[12]

## Conclusion

In conclusion, this research represents an *in vitro* evaluation useful for the selection of the microorganisms with the highest efficiency to remove $AFB_1$ in a wide range of mycotoxin concentration, able to tolerate salivary and GI conditions, and with potential beneficial properties on the host. Our results allow us to predict that *S. cerevisiae* 08 isolated from broiler feces, is the best candidate for future *in vivo* studies useful to prevent aflatoxicosis in poultry

industry, because it was able to survive salivary and GI tract conditions, it showed high efficiency in $AFB_1$ adsorption and good aggregation abilities, including all pathogenic bacteria assayed. Mycotoxin sequestration in the GI tract by adsorbing agents such as microorganisms could be a promising strategy to protect against the toxic effect of these secondary metabolites. Further quantitative *in vitro* and *in vivo* studies are required to evaluate the real impact of yeast-binding activity on the bioavailability of AFB in poultry. However, this study is useful not only in selecting efficient strains in terms of $AFB_1$-binding but also in providing an important contribution to research into microorganisms with potential probiotic effects on the host.

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