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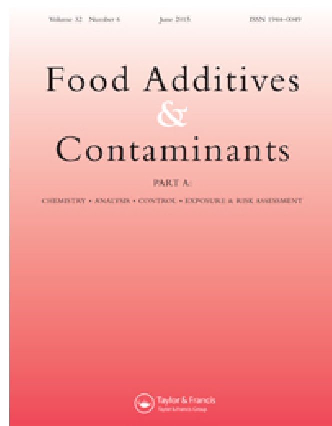

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Potentiation of the effect of a commercial animal feed additive mixed with different probiotic yeast strains on the adsorption of aflatoxin B_1

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Potentiation of the effect of a commercial animal feed additive mixed with different probiotic yeast strains on the adsorption of aflatoxin B_1

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This study potentiates the adsorbent effect for aflatoxin B_1 (AFB_1) of a commercial additive (CA) of animal feed, containing inactive lysate of three *Saccharomyces cerevisiae* strains, active enzymes, adsorbents and a selenium–amino acid complex, when the additive was mixed separately with three *S. cerevisiae* strains. Levels of AFB_1 of 20 and 50 ng g^{-1} were used to determine the binding capacity of different concentrations of CA alone and in the presence of yeast strains, as well as toxin desorption, under gastrointestinal conditions. The viability of yeasts in the presence of CA was evaluated. The results show that the CA did not affect the viability of the yeast strains assayed. CA alone showed a low percentage adsorption. At 20 and at 50 ng g^{-1}, CA was highly efficient in adsorbing AFB_1 when combined with RC016 and RC012 strains respectively. Desorption of AFB_1 by CA alone and in combination with the yeasts increased with increasing levels of CA. The results demonstrate the improvement of CA in AFB_1 adsorption once it is mixed with live yeasts.

Keywords: animal feed; AFB_1 binding; *Saccharomyces cerevisiae*

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Introduction

Mycotoxins are toxic secondary metabolites produced by certain species of moulds usually from the *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. The quality of the products used in animal feed can be affected as these feeds are ideal substrates for fungi growth, which, under favourable conditions, may favour the synthesis of mycotoxins. Production of these toxic metabolites can occur during the growth of the crop, during post-harvest storage or during the storage of the compounded feed (CAST 2003). Contamination of agricultural products and feed with mycotoxins is a problem of global importance due to its effects on public health: acute and chronic mycotoxicosis reducing life expectancy in developed countries as well as large economic losses in animal production. Food safety has become a very important topic worldwide since basic crops (cereals, dried fruits, fruits) can be colonised and infected by potential mycotoxin-producing fungi. Aflatoxins (AFs) are produced by *Aspergillus* spp.: *A. flavus*, *A. parasiticus* and *A. nomius* are the mayor AF-producing species (Frisvad et al. 2005). AFs in feed or feed ingredients are usually a mixture of four AFs with only slightly different chemical structures. The most prevalent and most toxic to animals is aflatoxin B_1 (AFB_1), which is mainly carcinogenic and hepatotoxic. The other forms are AFB_2, AFG_1 and AFG_2, which are included in the term 'total aflatoxins' (CAST 2003). AFs are heat stable, so it is very difficult to remove them during feed manufacture.

Several strategies for mycotoxin detoxification in feed have been developed, but most are not currently used due to the high costs of implementation or practical difficulties (Halász et al. 2009). Nowadays there is no available technology that can completely remove mycotoxins from food and feed. A promising alternative is biological detoxification using live and dead microorganisms that can adsorb mycotoxins into their cell wall components. The adsorption in biological systems is a reversible process, explained as AFB_1 binding to the external surface of the microorganism. Moreover, coupled with this phenomenon, some desorption or mycotoxin release can occur. This process is reversible with fast kinetics and can be regarded as physical adsorption (Yiannikouris et al. 2004; Bueno et al. 2007). The most important application of adsorption and desorption processes is the capacity to select the most efficient microorganism able to remove AFB_1.

The use of biological additives with the potential of preventing mycotoxicosis can reduce the severe impact on the morbidity and mortality in animals. They work by sequestering the toxins in the gastrointestinal (GI) tract by

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formation of insoluble complexes that are eliminated in the faeces. Yeasts, and especially *S. cerevisiae*, are one of the most important tools in biotechnology, since they are used in ethanol and beer production, and they are used to a high extent as a protein source in animal feed (Shetty & Jespersen 2006). The European Union (European Commission 70/524), Japanese pharmacopoeia (Nitta & Kobayashi 1999) and the USFDA have classified *S. cerevisiae* as a GRAS microorganism (generally recognised as safe). The cell wall of *S. cerevisiae* is made-up of mannoproteins, β 1–3 glucane and *n*-acetylglucosamine. The β 1–3 glucane and mannoproteins seem to be the binding sites for AFs, fumonisins, among others mycotoxins. It has been suggested that this may be the reason why the conditions of the GI tract could produce structural changes in the cell wall of yeast influencing mycotoxin adsorption and reducing bioavailability in the GI tract of the animal. The differences in the adsorption observed between different strains of *S. cerevisiae* when exposed to different conditions of the culture medium may be explained by the structural variability of the yeast cell wall (Yiannikouris et al. 2004). Several commercial products available in the market have proved to be efficient adsorbents when added to feed, producing inhibitory effects on different mycotoxins. However, some are only effective for *Fusarium* mycotoxins and have a low or no effect on AFs adsorption. Previous studies have demonstrated that *S. cerevisiae* strains isolated from healthy animals have the ability of sequestering AFB_1 under GI fluid conditions. These strains also have potential beneficial properties to be considered as probiotic microorganisms. Genotoxicity and cytotoxicity of *S. cerevisiae* strains were also evaluated *in vivo* in rats, with the results showing that the dietary administration of the strain does not induce genotoxicity or cytotoxicity to rats, enhancing its potential to be incorporated in the formulation of feed additives to increase animal productivity (Armando et al. 2011; Dogi et al. 2011; Gonzalez Pereyra et al. 2014). Companies dedicated to the production of additives to improve animal production require improvement in the adsorption capacity of AFB_1 coupled to health promotion of their commercial additives (CA). One promising approach is the use of live probiotic yeasts with mycotoxin adsorbent properties to achieve that goal. The aim of the present work was to potentiate the AFB_1 adsorbent effect of the CA when it was mixed separately with probiotic and mycotoxin-adsorbent strains of *S. cerevisiae* isolated from an animal environment.

Materials and methods

Yeast strains

Saccharomyces cerevisiae strains RC009, RC012 and RC016 were obtained from the collection centre at the Universidad Nacional de Río Cuarto, Argentina. They were previously isolated from feedstuffs (RC009 and RC012 strains) and pig gut (RC016 strains), and were characterised by molecular techniques. The selection of the strains was based on their capacity for *in vitro* mycotoxin binding and their probiotic proprieties (Armando et al. 2011; Armando, Dogi, et al. 2012; Armando, Pizzolitto, et al. 2012).

Commercial additive

The CA is composed by inactive lysate of three *S. cerevisiae* strains, active enzymes, adsorbents and a selenium–amino acid complex (supplied by Vetanco S.A., Vicente Lopez, Buenos Aires, Argentina).

Chemicals and media

The media used for the growth of *S. cerevisiae* strains in the assay was yeast extract, peptone and dextrose (YPD: 5 g yeast extract, 5 g peptone, 40 g dextrose, 20 g agar and 1000 ml water) broth and agar. Aflatoxin B_1 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Solid AFB_1 was suspended in benzene–acetonitrile (97:3 v/v) to obtain an AFB_1 concentration of 2 mg ml^{-1}. Solutions of AFB_1 at concentrations of 20 and 50 ng ml^{-1} were prepared. PBS was used in the binding assay; and artificial intestinal fluid (trypsin and chymiotrypsin 1 mg ml^{-1}; Fluka, St. Louis, MO, USA; and oxgall biliar salts 0.3% w/v) was used in the binding assay. Artificial intestinal fluid was adjusted to pH 8 with NaOH 5 N.

Viability assay of Saccharomyces cerevisiae *strains in the presence of commercial additive*

To determine cell viability, each *S. cerevisiae* strain was grown in YPD broth and incubated in agitation (150 rpm) at 25ºC for 24 h. Decimal dilutions were performed until a concentration of 10^7 cell ml^{-1} was reached. Later, 1 ml of yeast was transferred to an Erlenmeyer flask containing 100 ml of YPD broth mixed with CA in different concentrations if 50%, 75% and 100%. The cell suspension concentration was determined using a haemocytometer every 3 h for 12 h. Viability was confirmed by standard plate count method using YPD agar, according to Armando et al. (2011).

Aflatoxin B_1 binding capacity of commercial additive in the presence of yeasts strains under simulated gastrointestinal conditions

The binding capacity was assayed according to Peltonen et al. (2001), with modifications. Briefly, the assay was conducted using different CA concentrations (50%, 75% and 100%) and at simulated GI tract conditions. Cells (10^7 cell ml^{-1}) were washed twice with PBS and

incubated for 1 h at 37ºC in a shaking bath with 1 ml of artificial intestinal fluid (mentioned above) containing AFB_1 at a concentration of 20 ng g^{-1} (the maximum level allowed by international rules in dairy cattle feed) and 50 ng g^{-1} (exceeding the maximum level allowed by international rules). Then the cells were pelleted by centrifugation at 5000 rpm for 10 min at RT, and the supernatant containing unbound AFB_1 was collected and stored at −20ºC for HPLC analysis. Positive (artificial intestinal fluid plus mycotoxin) and negative (artificial intestinal fluid plus yeast cells) controls were included for all experiments. The experiment was conducted in triplicate.

Aflatoxin B_1 desorption assay in artificial gastrointestinal fluid

Pelleted cells/CA/bound AFB_1 (as described above) were suspended in 1 ml of artificial intestinal fluid and incubated 1 h at 37ºC with shaking. After that the microorganisms were pelleted by centrifugation and the supernatant containing the released AFB_1 was collected and stored at −20 C for later HPLC analysis. This process was repeated three times.

Quantification of aflatoxin B_1

Quantification was performed by HPLC (Waters e2695; Waters, Milford, MA, USA) according to the methodology proposed by Trucksess et al. (1994), with some modifications (Cole & Dorner 1994) and the AFB_1 solutions (standards) were prepared according to the AOAC (Rice et al. 1995). An aliquot (200 µl) of the samples was derivatised with 700 µl trifluoroacetic acid–acetic acid–water (20:10:70 v/v). Chromatographic separations were performed on a reversed-phase C18 column (150 × 4.6 mm i.d., 5 µm particle size; Phenomenex, Luna, Torrance, CA, USA). Water–methanol–acetonitrile (4:1:1 v/v) were used as the mobile phase at a flow rate of 1.5 ml min^{-1}. The fluorescence of AFB_1 derivatives was recorded at excitation and emission wavelengths of 360 and 460 nm, respectively (Waters 2475 multi λ fluorescence detector). The concentration of this toxin was quantified by correlating peak heights of sample extracts with those of standard curves. The LOD of the analytical method in artificial GI fluid was 0.1 ng g^{-1}.

Statistical analysis

Data were analysed by general linear and mixed model (GLMM) using InfoStat (version 2.03 for Windows 2012; University of Cordoba, Argentina) software. Yeast strains and CA adsorption of AFB_1 data were analysed by analysis of variance (ANOVA). Means and standard error (SE) were compared using the Fisher's protected least significant test (LSD) ($p < 0.0001$).

Results

The viability of the *S. cerevisiae* strain was evaluated alone and combined with the CA; results were obtained by the plate count method. None of the concentrations of CA influenced negatively the yeast strains assayed (data not shown).

Table 1 shows the statistical significance of yeasts, CA and their interactions on the percentage of AFB_1 adsorption. The results show that all variables assessed significantly influenced AFB_1 adsorption ($p < 0.0001$), at both tested AFB_1 levels.

In general, the percentage adsorption by the CA and *S. cerevisiae* strains individually, at different AFB_1 levels, showed variable behaviour (Table 2). *S. cerevisiae* RC016 at 20 and 50 ng g^{-1} and RC012 at 50 ng g^{-1} showed the highest percentage adsorption (71% and 82%, respectively), whilst RC009 showed the lowest percentage adsorption at both concentrations. The CA and *S. cerevisiae* RC009 did not show significant differences at both AFB_1 concentrations tested as well as RC012 at 20 ng g^{-1} AFB_1.

Figure 1 shows the percentage adsorption of AFB_1 (20 and 50 ng g^{-1}) and its desorption at different CA concentrations. The lower the concentration of CA, the higher the percentage adsorption, which varied between 0% and 45% at 20 ng g^{-1} AFB_1 and between 0% and 30% at 50 ng g^{-1} AFB_1. Desorption of AFB_1 did not exceed 20% at

Table 1. Statistical significance of the variables yeasts, CA and their interactions on aflatoxin B_1 adsorption percentages.

	Aflatoxin B_1					
	(20 ng g^{-1})			(50 ng g^{-1})		
Treatment	MS	*F*	*p*	MS	*F*	*p*
Yeasts	0.92	413.82	< 0.0001	14439.05	40524.22	< 0.0001
CA percentage	0.26	118.49	< 0.0001	1514.98	4251.89	< 0.0001
Yeasts*CA percentage	0.48	213.29	< 0.0001	1484.03	4165.03	< 0.0001

Note: MS, mean square; *F*-value, *F*-Snedecor.

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Table 2. Adsorption percentages by CA and *Saccharomyces cerevisiae* strains at different aflatoxin B_1 levels.

	Aflatoxin B_1					
	(20 ng g^{-1})			(50 ng g^{-1})		
Treatment	Media	SE	LSD test	Media	SE	LSD test
CA	23	± 5.68	ab	17	5.68	a
S. cerevisiae RC009	34	± 0.21	b	24	0.15	ab
S. cerevisiae RC012	33	± 0.22	b	82	0.15	d
S. cerevisiae RC016	71	± 0.21	cd	61	0.15	c

Note: Means with letters in common are not significantly different according to Fisher's protected least significant difference (LSD) test ($p < 0.05$). Binding level (ng g^{-1}) at each concentration of AFB_1 was statistically analysed separately (different letters indicate differences within each column).

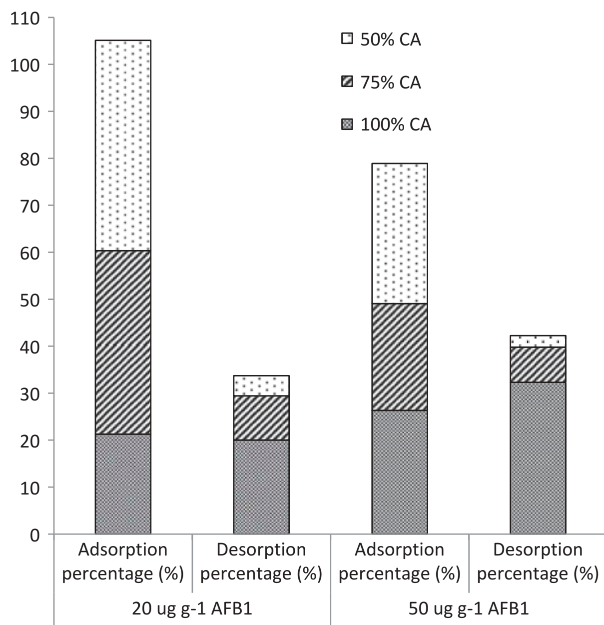

Figure 1. Aflatoxin B_1 (20 and 50 ng g^{-1}) adsorption and desorption percentages by different CA concentrations.

20 ng g^{-1} and 33% at 50 ng g^{-1}. The highest percentage adsorption was obtained at the highest percentage CA. Figure 2 shows the percentage AFB_1 (20 and 50 ng g^{-1}) adsorption and desorption at different percentages of CA by *S. cerevisiae* RC009, RC012 and RC016. The tested mixture between CA and RC009 showed no significant differences in its effectiveness at reducing AFB_1 levels at the different concentrations of CA and AFB_1 (Figure 2A). It is noteworthy that the presence of this yeast strain was effective at 50 ng g^{-1} of AFB_1; the adsorption percentages were higher than those obtained with the CA alone. Furthermore, this efficiency increased as the percentage of CA decreased. The mixture CA–*S. cerevisiae* RC009 was not effective at the lowest concentration of AFB_1 tested. Desorption rates were high reaching 35% with 20 ng g^{-1} of AFB_1 and 28% with 50 ng g^{-1} of AFB_1.

The tested mixture between the CA and *S. cerevisiae* RC012 proved to be effective at 50 ng g^{-1} of AFB_1 (Figure 2B). Adsorption rates increased as the concentration of CA increased. Desorption rates were lower than those observed with the mixture CA–*S. cerevisiae* RC009, reaching 18.8% with 20 ng g^{-1} of AFB_1 and 5.6% with 50 ng g^{-1} of AFB_1.

The tested mixture between CA and *S. cerevisiae* RC0016 was highly effective showing adsorption rates between 51.2% and 97.5% with 20 ng g^{-1} of AFB_1 and very small desorption percentages of between 6.3% and 8.0%. Adsorption increased with increasing concentrations CA. Furthermore, this mixture with 50 ng g^{-1} of AFB_1 showed no differences in adsorption to the different percentages of CA used and its effectiveness was significantly lower.

In general, the mixtures CA–*S. cerevisiae* RC012 (mainly at 50 ng g^{-1} AFB_1) and CA–*S. cerevisiae* RC016 (mainly at 50 AFB_1 ng g^{-1}) were the most efficient in their capacity to adsorb AFB_1 ($p < 0.0001$).

Discussion

The present study shows the improvement in the AFB_1 adsorption of a CA used as a mycotoxin binder in animal feed after the addition and mixture with *S. cerevisiae* strains isolated from an animal environment carried out at conditions found in the GI tract.

The CA used in this work consisted of an inactive lysate of *S. cerevisiae* strains plus a selenium–amino acid complex; some mineral components are necessarily added during manufacturing to avoid the formation of lumps (Fruhauf et al. 2012). There are many commercially available feed additives with the potential to reduce the toxicity of mycotoxins (Ramos et al. 1996; Huwig et al. 2001; Schatzmayr et al. 2006). Some of them are composed by bentonites, acid lactic bacteria, clay, clay plus live yeasts cells, clay plus dried yeasts cells, yeasts cell wall components, among others. Some of these additives have been more efficient reducing AFs than others, as a special type of clay that can adsorb from 98.0% to 99.55% (Ramos

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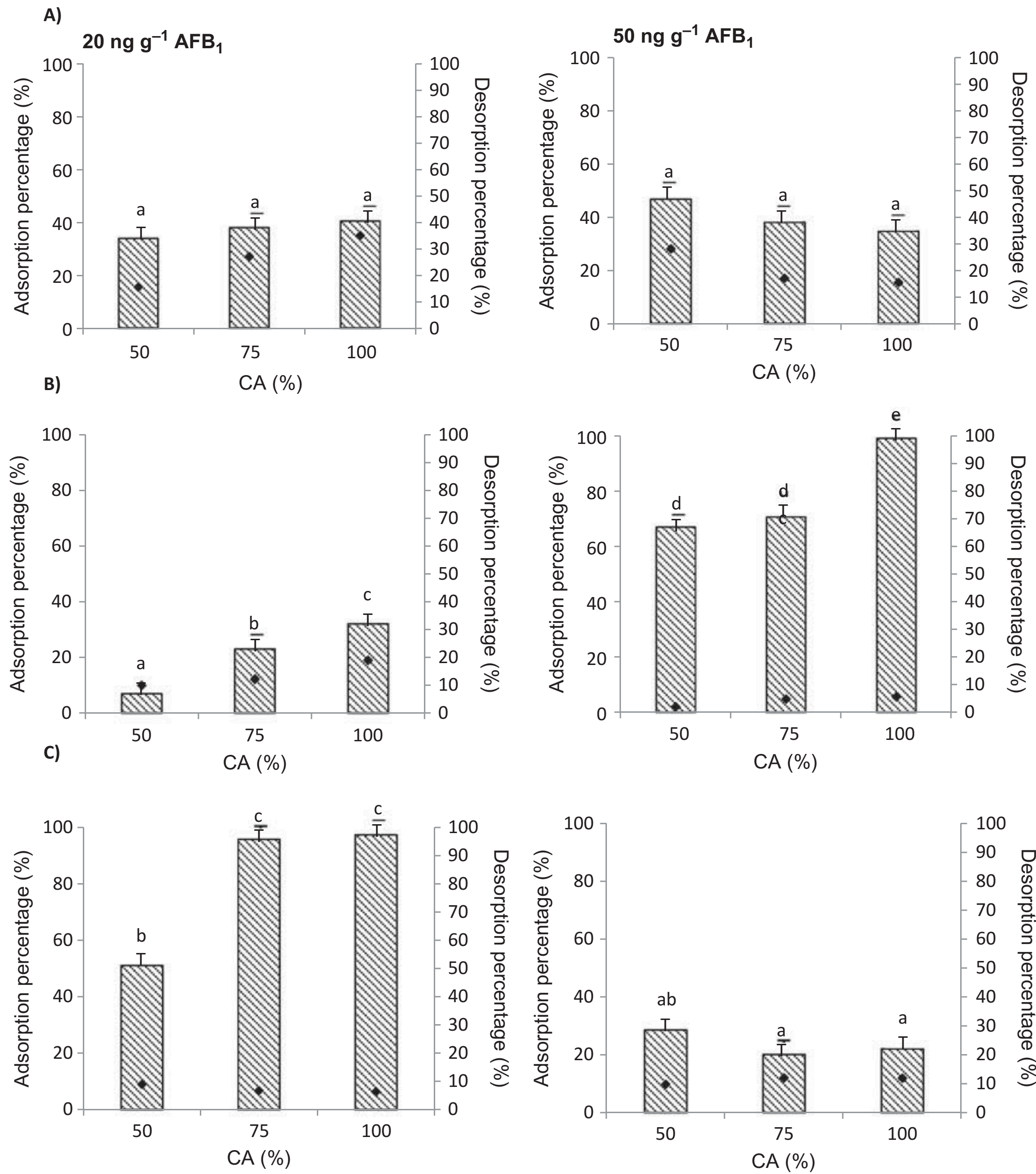

Figure 2. Aflatoxin B$_1$ (20 and 50 ng g^{-1}) adsorption and desorption percentages (± standard error (SE)) at different CA percentages by (A) *S. cerevisiae* RC009; (B) *S. cerevisiae* RC012; and (C) *S. cerevisiae* RC016. Letters in common are not significantly different according to Fishers protected LSD test ($p < 0.0001$). Statistical analysis was performed at the two studied AFB$_1$ concentrations for each yeast, separately.

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et al. 1996). On the other hand, it is well known that yeasts are capable of binding mycotoxins, including AFs, due to their cell wall components, mainly the glucomannans (El-Nezami et al. 1998; Haskard et al. 2000, 2001; Raju & Devegowda 2000; Peltonen et al. 2001; Lee et al. 2003; Shetty et al. 2007; Hernandez-Mendoza et al. 2009; Armando et al. 2011).

In the current study the CA had a percentage adsorption under 50%, and it was more efficient at adsorbing AFB$_1$ when it was evaluated in a mixture with some *S. cerevisiae* strains with demonstrated probiotic and mycotoxin reduction abilities (Armando et al. 2011) at different AFB$_1$ concentrations. The results demonstrated that CA combined with *S. cerevisiae* RC012 or RC016 at different conditions was highly efficient in AFB$_1$ adsorption. However, they did not show a similar behaviour between adsorption percentages and mycotoxin concentrations. Some authors have suggested an inverse relationship

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between percentage adsorption and mycotoxin concentrations: when AF concentration increases, the adsorption percentage declines. This behaviour may be due to the saturation of the adsorption sites in the *S. cerevisiae* cell and not to a molecular structure modification of the toxin (Fernandes Oliveira et al. 2013). Other researchers have suggested that increasing AFB_1 concentration in aqueous medium would not affect the percentage of AFB_1 binding. However, it can affect the binding speed (Rahayu et al. 2007). All these results show that AFB_1 adsorption is strain dependent. The adsorption isotherms are different for each microorganism. This implies that at the same toxin concentration sometimes saturation is achieved, while in other cases the saturation is achieved at higher toxin concentrations. This would explain why some mixtures in this work had the same adsorption at 20 and 50 ng g^{-1} AFB_1, while in other mixtures the adsorption increased with increasing concentrations of mycotoxin.

Regarding AFB_1 desorption, the results in the present work suggested that toxin binding is a reversible process. Also, it was demonstrated that AFB_1 was released in the same chemical form by the additive, yeast cell and from the mixture, so metabolic conversion of the toxin by cells did not take place. The reversibility of the process has been previously reported by Hernandez-Mendoza et al. (2009) who used PBS solution, showing around 60–70% of AFB_1 remained bound to the cells, suggesting that the toxin is attached to the bacteria by weak, non-covalent interactions that could be at least partially reversible. Pizzolitto et al. (2011) reported that after five washings with PBS, AFB_1 bound to different lactic acid bacteria cells was close to 50%, and the washing time did not change the percentage released when it varied from 1 to 60 min. The results obtained in the present work showed that the AFB_1 desorption of the CA in combination with *S. cerevisiae* strains increased as the CA increased. These results match those obtained by several authors who suggested that the cell wall components of yeasts could be involved in AFB_1 removal (Raju & Devegowda 2000; Haskard et al. 2001; Lahtinen et al. 2004; Karaman et al. 2005). The differences among desorption results may be attributed to differences of cell wall structure characteristics of each strain.

In conclusion, the results obtained in the present work are promising as regards the improvement of the CA assayed since the combination of live *S. cerevisiae* strains isolated from an animal environment increased the AFB_1 adsorption of the CA under GI conditions. Moreover, the assayed yeast strains have proved probiotic properties (Armando et al. 2011), so when the animal feed is supplemented with CA in addition mainly by live *S. cerevisiae* RC012 and RC016, besides the improved AFB_1 binding capacity, the additive will supply beneficial effects to the animal. These results are not only relevant to the specific studied CA and could have a wider application when used alone or in combination with other feed additives. Although the approach of using artificial GI fluid is widely accepted as proof of adsorption of AFs, biomarker studies with animals are being conducted in our laboratory and they will be required by the authorities that grant approval for feed additives.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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