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# Novel yeast isolated from broilers' feedstuff, gut and faeces as aflatoxin $B_1$ adsorbents

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## Abstract

**Aims:** To isolate and characterize native yeast strains from broilers' environment as feedstuff, faeces and gut, and to evaluate their binding capacity for aflatoxin $B_1$ ($AFB_1$).

**Methods and Results:** A total of nine yeast strains were isolated: three from feedstuff identified as *Pichia kudriavzevii* (2) and *Clavispora lusitaniae* (1), two from gut identified as *Candida tropicalis* and four from faeces identified as *Cl. lusitaniae* (3) and *Cyberlindnera fabianii* (1). $AFB_1$ binding percentages varied among yeast strains and with $AFB_1$ concentrations. To carry out adsorption studies, one strain from each genus and each origin was selected as follows: *Cl. lusitaniae* and *P. kudriavzevii* from feedstuff, *Cl. lusitaniae* and *Cy. fabianii* from faeces and *Ca. tropicalis* from gut. The most appropriate concentrations for cells and toxin were $10^7$ cells per ml and 100 ng $ml^{-1}$ of $AFB_1$ respectively. All the tested yeast strains showed similar adsorption capacities independently of the origin. The adsorption isotherm studies in all yeasts assayed showed behaviour of L type or Langmuir and a varied affinity for the toxin. The stability of the $AFB_1$–yeast complex demonstrated the irreversibility of the binding process.

**Conclusion:** Yeast strains tested in this study constitute potential $AFB_1$ adsorbents and they possess the advantage to be native from the avian environment.

**Significance and Impact of the Study:** This study makes a contribution to using native yeasts from broilers' environment for controlling chronic aflatoxicosis in avian production.

## Introduction

Mycotoxins are toxic secondary metabolites produced by fungi and represent a serious hazard to humans and animals (CAST 2003). Aflatoxins (AFs) are a group of naturally occurring mycotoxins produced by *Aspergillus* fungi, especially *Aspergillus flavus* and *Aspergillus parasiticus*. Twenty AFs have already been identified, with aflatoxin $B_1$ ($AFB_1$) being one of the most common and toxic compounds present in avian feed (Hussein and Brasel 2001). The toxicity of AFs in broilers has been widely investigated for their carcinogenic, mutagenic, teratogenic and growth inhibitory effects (Oğuz *et al.* 2000; Sur and Celik 2003; Magnoli *et al.* 2011a,b; Gutleba *et al.* 2015). Animals that consume AF-contaminated feed develop various health problems, including growth retardation, reduction in feed efficiency, and liver and kidney damage (Bintvihok 2002; Magnoli *et al.* 2011a; Bovo *et al.* 2015). Humans are exposed to AFs directly by the consumption of contaminated food or indirectly by the consumption

of products derived from animals that have consumed AF-contaminated feed (Bennett and Klich 2003).

Preventing mould growth and AF contamination in feed and feedstuffs is very important but when contamination cannot be prevented, decontamination of AF is needed before using these materials. Producers, researchers and governments aim to develop effective prevention management and decontamination technologies to minimize the toxic effects of AF. Practical and cost-effective methods of detoxifying AF-contaminated feed are in great demand. Besides preventive management, approaches have been employed including physical, chemical and biological treatments to detoxify AF in contaminated feeds and feedstuffs (Zaki *et al.* 2012). However, large-scale, practical and cost-effective methods for a complete detoxification of mycotoxin-containing feedstuffs are currently not available. The incorporation of non-nutritive substances such as sodium bentonite (NaB), hydrated sodium calcium aluminosilicates (HSCAS), among other similar dietary feed additives, have been reported in the literature (Magnoli *et al.* 2011a; Oğuz 2011). Adsorption onto various types of compounds (HSCAS, kaolin, silica-binding agent, bentonite, etc.) has been extensively studied in recent years (Magnoli *et al.* 2013). Although these products are widely and commercially available as feed additives for mycotoxin binding, they need to be incorporated at high levels and have side effects on some dietary nutrients, thus reducing the nutritional value of animal diets (Zain 2011).

Organic compounds such as yeast and yeast cell wall products have also been suggested to reduce aflatoxicosis in poultry (Freimund *et al.* 2003; Santin *et al.* 2003; Onwurah *et al.* 2013), and bacterial cell walls have been studied for their ability to complex with several mycotoxins without harming the environment or reducing the bioavailability of certain nutrients (El-Nezami *et al.* 2000, 2002; Roto *et al.* 2015). Other studies have indicated that *Saccharomyces cerevisiae* cell walls can be added to contaminated food or feeds to bind mycotoxins selectively (Yiannikouris *et al.* 2003, 2004). This allows a portion of the toxins to pass through the digestive tract without any negative effect on animals or carry-over to edible animal products such as milk, eggs or meat.

In the intestinal tract of broilers containing bacteria, fungi and protozoa, bacteria are the most predominant micro-organisms with a concentration in the ileum of $10^{8,9}$ CFU per g (colony-forming units) and in the caecum of $10^{10,11}$ CFU per g. It is estimated that the gastrointestinal tract of broilers has about $10^{13}$ bacteria and that only between 10 and 60% were able to be cultured and identified by conventional culture methods. The predominant species in the ileum correspond first to the genus *Lactobacillus* and then families Clostridiaceae, Streptococcaceae and Enterococcaceae. In contrast, the most abundant group detected in the caecum is Clostridiaceae and other micro-organisms in smaller proportion are *Pseudomonas* spp. and yeasts (Blajman *et al.* 2015). From the intestinal tracts of 35 out of 50 birds, the occurrences of individual yeast species were *Saccharomyces* spp. (31·03%), *Candida glabrata* (20·69%), *Candida tropicalis* (15·51%), *Candida albicans* (15·51%), *Candida fmata* and *Cryptococcus neoformans* (8·62%) (AL-Shimmery 2011).

The microbial status of intestinal tract of broilers depends on the composition of the diet, and the biota composition can influence overall bird performance. Additives based on natural viable micro-organisms (bacteria, yeasts or their combination) can help in establishing a beneficial intestinal population for animals and antagonising deleterious micro-organisms (Fowler *et al.* 2015; Roto *et al.* 2015).

In the poultry industry, *S. cerevisiae* has been used as a general performance promoter in poultry feeds and has recently been shown to have beneficial effects against $AFB_1$ exposure (Celyk *et al.* 2003; Stanley *et al.* 2004; Roto *et al.* 2015). Yeast cell walls consist almost entirely of proteins and carbohydrates. The carbohydrate fraction is composed primarily of glucose, mannose and *N*-acetyglucosamine. Glucans and mannans, the two main sugars, are found in about equal concentrations in *S. cerevisiae*. Yeast mannan chains of various sizes are exposed on the external surface and are linked to cell wall proteins (Evans and Dawson 2000). *In vitro* studies of Yiannikouris *et al.* (2004) have shown that $\beta$-D-glucans, and specifically (1–3)-$\beta$-D-glucans moderately branched with (1–6)-$\beta$-D-glucan chains from the cell wall of *S. cerevisiae* have shown affinity for zearalenone. These authors speculate that 'zearalenone-like' structures such as AFs, among others, could probably bind to $\beta$-glucans.

Many reports on the use of physically separated yeast cell walls obtained from brewery industry as feed additive in poultry diet have resulted in amelioration of toxic effects of AFs (Santin *et al.* 2003). Dried yeast and yeast cell walls added to rat-ration along with $AFB_1$ demonstrated a significant reduction in its toxicity (Baptista *et al.* 2004).

The aim of this study was to isolate and characterize native yeast strains from broilers' environment as feedstuff, faeces and gut, and to evaluate their binding capacity for $AFB_1$. This study was conducted for the further selection of potential $AFB_1$ adsorptive strains to be included in a novel anti-$AFB_1$ product for animal feed.

## Materials and methods

### Isolation of yeast strains

Yeast strains were isolated from feedstuff according to Fraga *et al.* (2007), from faeces according to Gusils *et al.*

(2002) and from broiler gut (jejunum and caecum). Samples (500 g feedstuff, broilers' caecum and jejunum and faeces) were collected from the Avian Research Unit of the Universidad Nacional de Río Cuarto (UNRC) and pooled according to each origin.

Ten grams of each feedstuff sample was added to 90 ml peptone water (0·1% w/v) and kept at room temperature for 30 min. This mixture was then shaken (30 min) and six decimal serial dilutions were carried out. One hundred microlitres of each dilution was inoculated in duplicate in Yeast extract-Peptone-Dextrose agar (YPD 5 g yeast extract, 5 g peptone, 40 g dextrose and 20 g agar, 1000 ml water). Samples of faeces (10 g) were processed as previously described for feed samples. Plates were incubated at 25°C for 48 h. Broiler caecum and jejunum from each sample were fragmented in pieces of 5 cm. Each segment was placed into 150 ml peptone water (0·1% w/v) and incubated at 37°C for 48 h. One hundred microlitres of each sample was inoculated in duplicate on YPD agar. Plates were incubated at 37°C for 48 h. All strains were kept in YPD agar at 4°C and maintained at −20°C in 30% (v/v) glycerol. Yeasts were propagated on Malt Extract Agar (MEA – 20 g malt extract, 20 g dextrose, 1 g peptone, 20 g agar, 1000 ml water) at 25°C for 24–48 h.

### Molecular identification of yeast strains

Species designation of yeast isolates was identified by molecular techniques by sequencing the 26S D1/D2 rRNA gene domain. Partial 26S-rRNA gene sequences (D1/D2 domains) were amplified using NL-1 (5′-GCATATCAAT AAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGT TTCAAGACGG-3′) primers according to Kurtzman and Robnett (2003). Briefly, amplification was performed using an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with an initial denaturation at 95°C for 5 min, followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 55·5°C for 2 min and extension at 72°C for 2 min. An additional extension at 72°C for 10 min was carried out at the end of 40 cycles. The amplified fragments were purified with a Pure Link PCR purification kit (Invitrogen by Life Technology, Carlsbad, CA) according to the manufacturer's instructions. Both strands of the rDNA region were sequenced with a Sanger capillary Sequencer 3130xl Genetic Analyzer (Applied Biosystems, Warrington, UK) using a Premix BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences obtained from both strands of each isolate were aligned and consensus sequence was obtained. The BLAST search (Basic Local Alignment) was used to compare the sequences obtained with databases of the National Centre for Biotechnology Information (NCBI). Identification was considered correct when gene sequences showed 100% identity.

### Production and purification of aflatoxin $B_1$

Aflatoxins for *in vitro* assays were produced via the fermentation of milled corn by *A. parasiticus* NRRL 3000. The sterile substrate placed in Erlenmeyer flasks was inoculated with 2 ml of the mould's aqueous suspension containing $10^6$ spores per ml. Cultures were allowed to grow for 7 days at 25°C in the dark. On the 7th day, Erlenmeyer flasks were autoclaved and the culture was material dried at 40°C in a forced air oven for 48 h. AFs were extracted with chloroform and purified by flash chromatography following the procedure described in AOAC (1994).

The total AF content in the purified extract of the culture was determined by high performance liquid chromatography (HPLC) according to Trucksess *et al.* (1994) and AOAC (1994). The purity of the purified extract is demonstrated in Fig. 1a (standard) and Fig. 1b (purified extract).

### Yeast inoculum preparation

The inoculum of each yeast strain was prepared from a 37°C overnight culture in YPD and harvested by centrifugation. The cells were then resuspended in peptone water (0·1% w/v) and serial decimal dilutions were done to obtain $10^4$, $10^5$, $10^6$ and $10^7$ cells per ml. The cell suspension concentration was determined using a haemocytometer. Viability was confirmed by the standard plate count method using YPD agar.

### Aflatoxin $B_1$ binding assay

The best concentration of cells to carry out adsorption studies was first selected among the decimal dilutions prepared above with a known mycotoxin concentration (10 ng $ml^{-1}$).

Then, different $AFB_1$ working solutions with concentrations of 10, 20, 30, 50 and 100 ng $ml^{-1}$ were prepared for each experimental condition as follows. Aliquots of 1 ml of yeast ($10^7$ cells per ml) were washed twice with PBS and incubated for 1 h at 37 ± 0·5°C, in a shaking bath with 1 ml of buffer pH 2, centrifuged for 15 min at 16 000 ***g*** at room temperature. One millilitre of buffer (pH 6) was then added separately to each $AFB_1$ concentration and incubated for 1 h at 37 ± 0·5°C in a shaking bath. The cells were then pelleted by centrifugation for 15 min at 16 000 ***g*** at room temperature. The supernatant containing unbound mycotoxin was collected for HPLC analysis, whereas the pellets containing bound

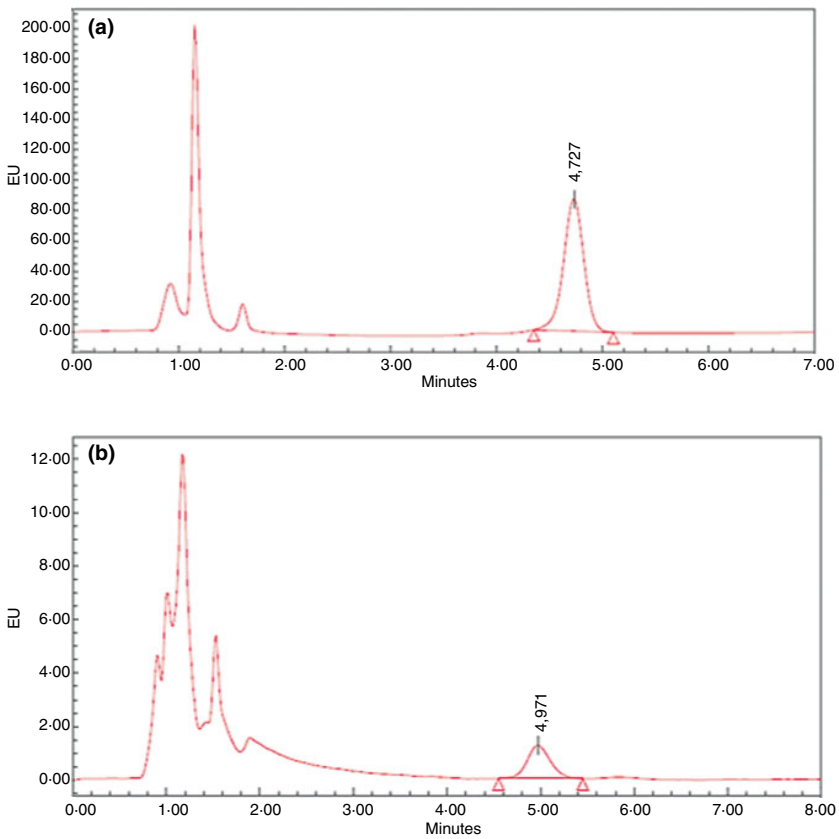


**Figure 1** A sample HPLC chromatogram of (a) standard and (b) purified extract.[Colour figure can be viewed at wileyonlinelibrary.com]

mycotoxins were maintained at 4°C for desorption assay. Controls of $AFB_1$ and blanks with yeasts were also included for comparison at each isothermal assay. The adsorbed $AFB_1$ was calculated from the depletion of the toxin in the supernatant after incubation. Adsorption experiments at each toxin concentration were performed in triplicate.

Buffer at pH 2 was prepared by mixing 62·5 ml of 0·2 mol $l^{-1}$ sodium chloride with 16·25 ml of 0·2 mol $l^{-1}$ hydrochloric acid. The final pH value was adjusted to make up the volume to 250 ml. Buffer at pH 6 was prepared by mixing 125 ml of potassium dibasic phosphate (0·1 mol $l^{-1}$) with 14 ml of sodium hydroxide (0·1 mol $l^{-1}$) following the procedure described above.

### Detection and quantification of aflatoxin $B_1$

Detection and quantification of $AFB_1$ were performed on HPLC (Waters e2695; Waters, Milford, MA) and fluorescence was detected according to the methodology proposed by Trucksess *et al.* (1994). The excitation range and emission wavelength ranges were 305–395 and 430–470 nm respectively. A C18 Luna Phenomenex column (150 mm × 4·6 mm, 5 $\mu$m; Phenomenex Inc., Torrance, CA) with the corresponding precolumn was used. The mobile phase was methanol/acetonitrile/water (1 : 1 : 4 v/v/v) at a flow rate of 1·5 ml $min^{-1}$ and precolumn derivatization. For derivatization, aliquots (200 $\mu$l) were allowed to react with 700 $\mu$l of acetic acid/trifluoroacetic acid/water (20 : 10 : 70) solution. The tube was allowed to stand for 9 min at 65°C in the dark (AOAC 1994). The AFs in the extract were mainly $AFB_1$ and $AFG_1$. The calibration curve was made with a mixture of solutions of $AFB_1$, $AFG_1$, $AFG_2$ and $AFB_2$ (purity >99%; Sigma Chemical Co., St. Louis, MO) with concentrations of 2·06, 1·99, 0·520 and 0·508 $\mu$g $ml^{-1}$ respectively. The concentrations of chromatographic standards were 5, 10 and 15 ng $ml^{-1}$ of $AFB_1$. Standard solutions for the calibration curves were prepared daily.

### Aflatoxin $B_1$ desorption assays

Pellets containing bounded mycotoxins previously obtained were used for desorption assays. These pellets were resuspended in 1 ml of the extraction solvents; buffers at pH 2 and 6 were prepared as described above and thoroughly mixed for 1 h. Afterwards, they were centrifuged for adsorbate determination in the supernatant. The procedure was performed with each of the extraction solvents. The percentage of adsorbate released was

determined by comparing the amount of adsorbate in the supernatant with the initial adsorbed amount for each assay. Two replicates for each assay were assessed.

### Statistical analyses

*Aflatoxin $B_1$ adsorption isotherms*

Curve fitting and data processing: Data were analysed by a theoretical model of Langmuir (L). Curves representing the amount of bounded $AFB_1$ as a function of the concentration of the free toxin in equilibrium after adsorption were plotted. The selection was made following the criteria suggested by Hinz (2001). Mathematical expression and parameters of model are shown in Table 1. The surface excess of $AFB_1$ ($\Gamma_{AFB1}$) in mg of $AFB_1$ per g of adsorbent was determined according to Eqn (1):

$$\beta_{Ads} = \left[\frac{\Gamma_{Ads}}{(\Gamma_{Ads,max} - \Gamma_{Ads})[Ads]}\right] \exp(-2\alpha\Gamma/\Gamma_{max}) \quad (1)$$

$\Gamma_{Ads}$ is the number of sites occupied by the toxin and $\Gamma_{Ads,\ max}$ are the largest number of sites occupied by the toxin in the yeast cell surface (mg $g^{-1}$), [Ads] is $AFB_{1c}$ concentration in the equilibrium, $\beta_{Ads}$ is the Langmuir adsorption constant (L). '$\alpha$' is the Langmuir parameter that measures the interaction between adsorbed $AFB_1$ molecules. A nonlinear least squares method, with a tolerance limit of 0·05, was used for curve fitting.

### Binding levels of aflatoxin $B_1$

Data (% and ng $ml^{-1}$) were analysed by generalized linear mixed models (GLMM) using INFOSTAT (ver. 2.03 for Windows 2012; University of Córdoba, Córdoba, Argentina) software. Data from yeast strain adsorption of $AFB_1$ assay were analysed by analysis of variance. Assumptions of normality and homogeneity were revised; untransformed data showed stabilized variance, and for this reason, no further transformation was applied. Means and standard error were compared using the Fisher's protected least significant test ($P < 0{\cdot}0001$).

**Table 1** Theoretical adsorption model, mathematical equations and adjusting parameters

| Models | Mathematical expression | Parameters |
|---|---|---|
| Langmuir | $\beta = \Gamma/(\Gamma_{max} - \Gamma)[AFB_1]$ | $\Gamma_{max}$, $\beta$ |

$AFB_1$, aflatoxin $B_1$.

$\Gamma$ is $AFB_1$ surface excess per gram of yeast; $[AFB_1]$ is the residual toxin at equilibrium; $\Gamma_{max}$ is the largest number of sites occupied by the toxin in the yeast cell surface; $\beta$ is the Langmuir adsorption constant (L).

## Results

### Isolation and molecular characterization of yeast strains

Isolated yeast strains and sources are shown in Table 2. A total of nine yeast strains were isolated from different samples: three from feedstuff identified as *Pichia kudriavzevii* (2) (ex *Issatchenkia orientalis*) and *Clavispora lusitaniae* (1) (anamorph *Candida lusitaniae*), two from gut identified as *Ca. tropicalis* and four from faeces identified as *Cl. lusitaniae* (3) and *Cyberlindnera fabianii* (ex *Pichia fabianii*) (1). To carry out adsorption studies, one strain from each genus and each origin was selected as follows: *Cl. lusitaniae* and *P. kudriavzevii* from feedstuff, *Cl. lusitaniae* and *Cy. fabianii* from faeces and *Ca. tropicalis* from gut. Their identifications were considered correct when gene sequences showed identities at 100% level.

### Aflatoxin $B_1$ binding

The ability of these yeast strains to bind $AFB_1$ *in vitro* is summarized in Table 3.

Aflatoxin $B_1$ binding percentages varied among yeast strains and with $AFB_1$ concentrations. The binding level (% and ng $ml^{-1}$) increased with increasing cell concentrations; for example, for *P. kudriavzevii*, at $10^5$ cells per ml, the binding level was 12·6% and 3·4 ± 1·2 ng $ml^{-1}$, whereas at higher cell concentrations ($10^7$ cells per ml), the binding level was higher (30·6% and 20·7 ± 1·3 ng $ml^{-1}$). The same behaviour was observed throughout the experiment. According to these results, and considering the tested cell concentrations, the most appropriate concentration for adsorption studies was $10^7$ cells per ml. On the contrary, the adsorption capacity of the tested strains increased with increasing toxin concentration; for example, *P. kudriavzevii* showed a binding level of 10·1–20·7 ng $ml^{-1}$ when 50 and 100 ng $ml^{-1}$ of $AFB_1$ were used respectively. All the tested yeast strains showed similar adsorption capacities independently of the origin. However, *P. kudriavzevii* and *Cl. lusitaniae* isolated from feedstuff showed the better behaviour. Based on the obtained results, adsorption isotherm

**Table 2** Yeast species isolated from feedstuff, broiler gut and faeces

| Source | Molecular identification (number of strains) |
|---|---|
| Feedstuff | *Pichia kudriavzevii* (2) |
| | *Clavispora lusitaniae* |
| Gut | *Candida tropicalis* (2) |
| Faeces | *Cl. lusitaniae* (3) |
| | *Cyberlindnera fabianii* (1) |

**Table 3** Aflatoxin $B_1$ ($AFB_1$) binding percentage (%) in different yeast strains (cells per ml) and at different toxin concentrations (ng $ml^{-1}$)

| | | | $AFB_1$ (ng $ml^{-1}$) | | | |
|---|---|---|---|---|---|---|
| | | | 50 | | 100 | |
| | | | Binding level | | | |
| Source | Yeast strain | Concentration (cells per ml) | % | ng $ml^{-1}$ | % | ng $ml^{-1}$ |
| Faeces | *Clavispora lusitaniae* | $10^5$ | 12·95 | $3·41 \pm 1·12^{ac}$ | 16·70 | $6·51 \pm 1·98^{a}$ |
| | | $10^6$ | 14·49 | $4·71 \pm 1·11^{b}$ | 19·91 | $9·81 \pm 1·90^{b}$ |
| | | $10^7$ | 15·09 | $5·51 \pm 1·11^{c}$ | 25·72 | $15·12 \pm 0·42^{c}$ |
| | *Cyberlindnera fabianii* | $10^5$ | 12·50 | $3·32 \pm 0·98^{a}$ | 16·89 | $7·10 \pm 0·70^{a}$ |
| | | $10^6$ | 15·89 | $6·00 \pm 1·13^{b}$ | 22·34 | $12·51 \pm 0·34^{b}$ |
| | | $10^7$ | 20·12 | $10·1 \pm 1·11^{c}$ | 25·41 | $18·22 \pm 0·69^{c}$ |
| Feedstuff | *Cl. lusitaniae* | $10^5$ | 12·26 | $3·30 \pm 0·73^{a}$ | 25·95 | $15·23 \pm 1·40^{a}$ |
| | | $10^6$ | 14·70 | $4·51 \pm 0·11^{b}$ | 29·37 | $19·12 \pm 0·64^{b}$ |
| | | $10^7$ | 15·55 | $5·22 \pm 1·58^{c}$ | 30·05 | $20·11 \pm 0·24^{c}$ |
| | *Pichia kudriavzevii* | $10^5$ | 12·57 | $3·43 \pm 1·23^{ac}$ | 22·99 | $12·88 \pm 1·71^{a}$ |
| | | $10^6$ | 13·99 | $3·81 \pm 1·15^{ab}$ | 25·94 | $15·98 \pm 1·45^{b}$ |
| | | $10^7$ | 20·86 | $10·11 \pm 1·21^{c}$ | 30·62 | $20·68 \pm 1·27^{c}$ |
| Gut | *Candida tropicalis* | $10^5$ | 12·95 | $2·45 \pm 1·12^{a}$ | 18·56 | $11·88 \pm 1·31^{a}$ |
| | | $10^6$ | 13·65 | $3·50 \pm 1·11^{b}$ | 22·98 | $14·98 \pm 1·25^{b}$ |
| | | $10^7$ | 20·65 | $9·10 \pm 0·98^{c}$ | 28·90 | $19·58 \pm 1·01^{c}$ |

Mean ± SD (standard deviation) $n = 3$.
Values with the same letter are not significantly different according to Fisher's protected least significant test ($P < 0·0001$). Statistical analysis compared the means obtained from each yeast strain separately.

studies were performed with a yeast concentration of $10^7$ cells per ml.

Figure 2 represents the amount of bounded $AFB_1$ as a function of the free toxin concentration and shows that curves were 'L'-shaped and could be fitted according to the Langmuir model, indicating a finite number of equivalent adsorption sites on the absorbent surface (Giles *et al.* 1974a,b). Table 4 shows the adjusting parameters of theoretical model Langmuir (L). All the yeasts assayed showed L-type behaviour and varied affinity for the toxin through the affinity constant ($\beta$). The values of the adsorption constant ($\beta$) showed a moderate *in vitro* affinity between yeasts and the toxin for all the strains evaluated at the assayed pHs. The highest $AFB_1$ affinity was observed for *Cl. lusitaniae* from feedstuff ($0·032 \pm 0·004$ mol $l^{-1}$). The lowest value was observed for *P. kudriavzevii* from feedstuff ($0·005 \pm 0·002$ mol $l^{-1}$). Among the strains isolated from faeces, *Cy. fabianii* and *Ca. tropicalis* isolated from the gut showed a moderate affinity for the toxin ($0·011 \pm 0·001$ mol $l^{-1}$ and $0·017 \pm 0·009$ mol $l^{-1}$ respectively).

As can be observed in Table 4, the two highest values of surface excess ($\Gamma_{max}$) were observed for *P. kudriavzevii* ($19·9 \pm 0·7$ mg $g^{-1}$) and *Cl. lusitaniae* ($16·1 \pm 1·7$ mg $g^{-1}$) from feedstuff, while the lowest values were observed for *Cy. fabianii* ($3·9 \pm 0·0$ mg $g^{-1}$) and *Cl. lusitaniae* ($6·3 \pm 2·0$ mg $g^{-1}$) from faeces and *Ca. tropicalis* ($4·5 \pm 0·1$ mg $g^{-1}$) from the gut.

### Aflatoxin $B_1$ desorption assays

The stability of the $AFB_1$–yeast complex was studied by repeated washings of the cellular pellets that previously bound with the mycotoxin, with water, buffer at pH 2 and 6. The results showed that the toxin was not present in the supernatant of all the tested yeast strains, thus demonstrating the irreversibility of the binding process (data not shown).

The derivatized $AFB_1$ retention time was 4·7 min and the limit of detection was 0·001 $\mu$g $ml^{-1}$.

## Discussion

In the poultry industry, *S. cerevisiae* has been used as a general performance promoter and has recently been shown to have beneficial effects against $AFB_1$ exposure (Celyk *et al.* 2003). Thus, the present work reports the isolation and selection of yeast strains—from broilers' environment—that were able to bind $AFB_1$, to tolerate gastrointestinal conditions and to demonstrate their $AFB_1$ adsorption capacity *in vitro*. *Clavispora lusitaniae*, *P. kudriavzevii*, *Cy. fabianii* and *Ca. tropicalis* were isolated from broiler natural environments. They were selected based on their $AFB_1$-binding ability and resistance to gastrointestinal conditions *in vitro*. Many commercially available feed additives with the potential to reduce the toxicity of mycotoxins have been reported (Ramos *et al.*

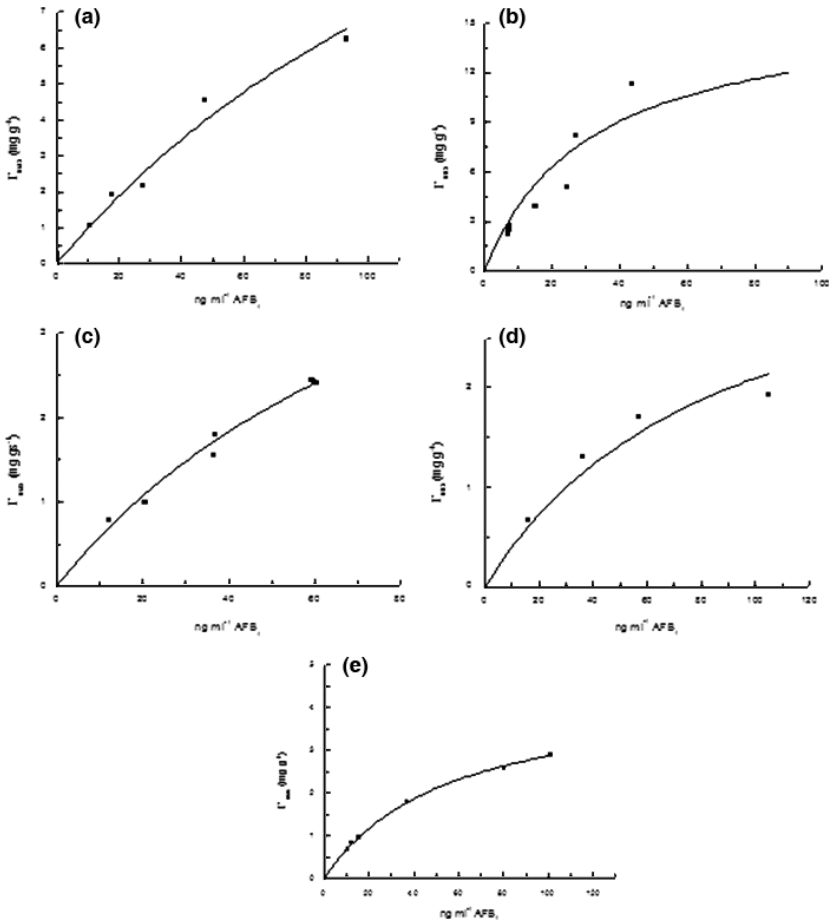


**Figure 2** The amount of bound aflatoxin $B_1$ is represented as a function of the free toxin concentration. (a) *Clavispora lusitaniae* and (b) *Pichia kudriavzevii* from feedstuff; (c) *Cl. lusitaniae* and (d) *Cyberlindnera fabianii* from faeces; (e) *Candida tropicalis* from gut.

**Table 4** Adjusting parameters

| Source | Strain | Experimental conditions | $\Gamma_{max}$, mg $g^{-1}$ | $\beta/(\text{mol } l^{-1})^{-1}$ | $R^2$ | α |
|---|---|---|---|---|---|---|
| Faeces | *Clavispora lusitaniae* | Buffer pH 2 and pH 6 | 6·32 ± 2·00 | 0·010 ± 0·005 | 0·98 | 0 |
| | *Cyberlindnera fabiani* | | 3·96 ± 0·01 | 0·011 ± 0·001 | 0·93 | 0 |
| Feedstuff | *Cl. lusitaniae* | | 16·14 ± 1·75 | 0·032 ± 0·004 | 0·98 | 0 |
| | *Pichia kudriavzevii* | | 19·92 ± 0·71 | 0·005 ± 0·002 | 0·98 | 0 |
| Gut | *Candida tropicalis* | | 4·48 ± 0·11 | 0·017 ± 0·009 | 0·99 | 0 |

$\Gamma_{max}$ is the surface excess at saturation per gram of yeast; $\beta$ is the Langmuir adsorption constant (L) ($mol^{-1}$); '$\alpha$' is the Langmuir parameter that measures the interaction between adsorbed $AFB_1$ molecules; $R^2$ determines the model quality.

1996; Huwig *et al.* 2001; Schatzmayr *et al.* 2006; Armando *et al.* 2011a,b; Magnoli *et al.* 2013, 2014). They were composed of bentonites, lactic acid bacteria, clay, clay plus live yeast cells, clay plus dried yeast cells, yeast cell wall components, and have demonstrated different efficiencies in reducing AFs among them (Ramos *et al.* 1996; Bueno *et al.* 2007; Armando *et al.* 2011a,b; Magnoli *et al.* 2013, 2014; Poloni *et al.* 2015). The union of binders with toxins reduces their availability and consequently, the absorption of the toxin in the gastrointestinal tract. 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 and Devegowda 2000; Peltonen *et al.* 2001; Lee *et al.* 2003; Shetty *et al.* 2007; Hernandez-Mendoza *et al.* 2009; Armando *et al.* 2011b; Poloni *et al.* 2015). Recently, new products based on yeast cell wall are available on the market, especially for bird feeds (Karaman *et al.* 2005; Fowler *et al.* 2015). Some authors consider that native strains have advantages over the foreign ones. The microbiota of the gastrointestinal tract play a crucial role in the anatomical, physiological and immunological development of the host. They have to be safe for the host, genetically stable and capable of surviving

passage through the gastrointestinal tract. The biota of a specific community are well adapted to the environment of their intestine, whereas foreign biota must compete with these microbes as they originated from a population having different feed habitats (Duary *et al.* 2012). No studies have reported the use of native yeasts from broiler natural environments or the use of strains belonging to *Pichia, Clavispora*, *Candida* or *Cyberlindnera* genera.

Results obtained in this study showed that the percentage of $AFB_1$ binding ranged from 25·7 to 30·6%, according to the $AFB_1$ concentration tested. *Pichia kudriavzevii* showed the highest binding percentage at 100 ng $ml^{-1}$ of $AFB_1$, followed by *Cl. lusitaniae* strain. This concentration or a lower concentration can be naturally found in feed.

Other studies have demonstrated binding levels of $AFB_1$ that ranged from 5 to 84% and 0·6 to 46% by micro-organisms such as lactobacilli involved in fermented dairy foods (Peltonen *et al.* 2000, 2001; Haskard *et al.* 2001). Although all the tested strains in this study were capable of binding $AFB_1$, the binding level appears to vary among strains, indicating the strain-specific binding nature. Similarly, other studies have reported a wide range of genera, species and strains such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Lactococcus* spp. with specific $AFB_1$ binding capacities (Peltonen *et al.* 2000, 2001; Haskard *et al.* 2001; Lee *et al.* 2003; Shahin 2007; Shetty *et al.* 2007). In agreement with our results, Armando *et al.* (2011b) demonstrated higher binding levels of $AFB_1$ using yeasts isolated from faeces (*Candida* spp. and *Cl. lusitaniae*) than those present in the gut of pigs (*S. cerevisiae* and *I. orientalis*). In addition, Shahin (2007) and El-Nezami *et al.* (1998) showed that bacterial concentration influences $AFB_1$ removal; approximately a minimum of $10^9$ cells per ml is required for significant $AFB_1$ removal (13–50%), whereas a concentration of $10^{10}$ cells per ml is capable of reducing the $AFB_1$ level to <0·1–13%. These results agree with those reported in this study where the influence of yeast concentration ($10^7$ cells per ml) on $AFB_1$ removal exerted an effective $AFB_1$ adsorption. This fact is probably due to a larger cell surface associated with higher total sites per cell according to the model proposed by Bueno *et al.* (2007). Yeast cell is larger than a bacterial cell and it has more sites capable of binding $AFB_1$. El-Nezami *et al.* (1998) found that the amount of $AFB_1$ removed increased with increasing concentrations of $AFB_1$, but the removed percentage was not significantly different. In concordance with these authors, we found that adsorption increased as $AFB_1$ concentration increased and have demonstrated that mycotoxin adsorption in biological systems is a reversible process that can be characterized as a chemical equilibrium. In agreement with this study, Ramos *et al.* (1996) and Grant and Phillips (1998) showed that the adsorption is a concentration-dependent process influenced by mycotoxin concentration, the amount of adsorbent and the relative affinity of the adsorbent for the mycotoxin. Furthermore, the adsorption process in this study was irreversible for the tested strains.

Aflatoxin adsorption is influenced by pH and phosphate concentration in an aqueous environment. It was reported that maximum binding occurs at a pH of approx. 4·0 and in 0·5 mol $l^{-1}$ phosphate. Both optimal pH and phosphate concentration are consistent with those found in the gastrointestinal tract and suggest that the conditions in the gastrointestinal tract would enhance adsorption and not decrease the mycotoxin-adsorbent interactions (Dawson *et al.* 2001). In this way, it was found that gastrointestinal conditions could not affect the $AFB_1$ adsorption percentage of the tested yeast strains. These results suggest that these strains could reduce $AFB_1$ bioavailability at the gut level. Future studies should be conducted to confirm the statement of irreversibility of the binding process. In this work, it was proved by washing the surface of the cells; however, food processing and digestion could theoretically release $AFB_1$.

It is important to point out that the effects of $AFB_1$ on the yeasts itself were not determined in this study; however, Dogi *et al.* (2013) demonstrated a significant increase in *S. cerevisiae* RC016 cell diameters in the presence of $AFB_1$. This behaviour suggested an advantage, as a larger cell with a larger surface and a higher number of exposed binding sites would be able to act more efficiently as a mycotoxin adsorbent.

The advantage of *in vitro* models is the possibility of rapidly screening the mycotoxin binding ability of different yeast strains, thus enabling preselection. Future *in vivo* assays should be performed in order to study the influence of interactions among toxins and feed components, broilers' microbiota and the effect of digestive enzymes on binder–toxin complexes.

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## Conflict of Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Sequences obtained from nine isolates were reliable and accurate