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Binding of Aflatoxin B_1 to Lactic Acid Bacteria and *Saccharomyces cerevisiae* in vitro: A Useful Model to Determine the Most Efficient Microorganism

Romina P. Pizzolitto, Dante J. Bueno, María R. Armando, Lilia Cavaglieri, Ana M. Dalcero and Mario A. Salvano
Universidad Nacional de Río Cuarto
Argentina

1. Introduction

Mycotoxins are toxic fungal metabolites found as contaminants in many agricultural products. Feeds contaminated with mycotoxins have a health risk to animals and, as a consequence, may cause big economical losses due to the low efficacy of animal husbandry (Richard, 2007). In addition, directly or indirectly (animal by-products) contaminated foods may also have a health risk to humans (CAST, 2003; Hussein & Brasel, 2001; Wild, 2007).

Aflatoxins (AFs), a group of potent mycotoxins with mutagnic, carcinogenic, teratogenic, hepatotoxic and immunosupresive properties, are of particular importance because of their major occurrence and adverse effects on animal and human health, generalized as "aflatoxicosis" (CAST, 2003; Hussein & Brasel, 2001; Magnoli et al., 2011).

The AFs are produced by genus *Aspergillus*, mainly *A. flavus, A. parasiticus* and *A. nomius*, that grow on a variety of raw material during growth, harvest, storage and transportation of for example, the cereal used in the preparation of food and feed commodities (Ito et al., 2001; Kurtzman et al., 1987; Payne, 1998; Pereyra et al., 2010).

The investigation of strategies to prevent the presence of AFs in foods, as well as, to eliminate, inactivate or reduce the bio-availability of these mycotoxins in contaminated products include physical, chemical, and biological methods (Bueno et al., 2001; CAST, 2003; Kabak et al., 2006). Limitations such as the loss of nutritional and sensory qualities of the product, the expensive equipment required for these techniques and the impossibility to guarantee the desired results, have allowed us to consider the hipothesis that foods and feeds can always be potentially contaminated with aflatoxins. For instance, in the poultry industry aflatoxin B_1 (AFB_1) is almost an unavoidable feed contaminant and levels from 0-200 ng/g have been reported (Dalcero et al., 1997).

On the other hand, it is known that lactic acid bacteria (LAB) and some yeast, principally *Saccharomyces cerevisiae*, are capable to bind AFs in liquid media, apparently to cell wall components, polysaccharides and peptidoglycans of LAB (Haskard et al., 2001; Latinen et al., 2004) and glucomannans of yeast (Karaman et al., 2005; Raju & Devegowda, 2000) and

therefore could be used as potential mycotoxin decontaminating (Armando et al., 2011; El-Nezami et al., 1998; Haskard et al., 2000, 2001; Hernandez-Mendoza et al., 2009; Lee et al., 2003; Peltonen et al., 2001; Shetty et al., 2007). The inclusion of appropriate microorganisms in the contaminated diet could prevent the absorption of mycotoxins during their passage in the gastrointestinal tract and eliminated in the faeces (Bueno et al., 2007; El-Nezami et al., 2000; Gratz et al., 2004, 2007). Moreover, Kankaanpää et al. (2000) showed that the binding of AFB_1 to the surface of LAB reduced their adhesive properties, and the accumulation of aflatoxins in the intestine may therefore be reduced via the increased excretion of an aflatoxin-bacteria complex.

These considerations encouraged the recent emphasis on biological methods, but mainly focused on preventing AFs absorption in the gastrointestinal tract of the consumers, including these microorganisms in the diet and so prevent the aflatoxicosis effects.

The first step in this direction is the selection of the most efficient microorganism for AFB_1 removing and while many researchers have assayed LAB and yeast with AFB_1 binding abilities (Ciegler et al., 1966; El-Nezami et al., 1998; Gourama & Bullerman, 1995; Haskard et al., 2001; Line et al., 1994; Oatley et al., 2000) no clear mechanism for this effect has been provided. Thus, this selection frequently is performed using a single concentration of AFB_1, but we demonstrated that the microorganism efficiency may change when the mycotoxin concentration is modified (Bueno et al., 2007; Pizzolitto, 2011), therefore the microorganism selected could not be the most competent.

In this context, we investigated the nature of the interaction between different microorganisms and AFB_1 molecule, which led us to develop a model to explain the binding of AFB_1 by LAB and *Saccharomyces cerevisiae* strains. This model allows an estimation of two important parameters related to a microorganism's capacity for dietary decontamination: the number of binding sites for AFB_1 in the surface microorganism (M) and the equilibrium constant of the process involved (K_{eq}), both of them are useful in the selection of the most suitable microorganism in a wide range of AFB_1 concentration (Bueno et al., 2007).

In adittion, studies of viability of the microorganisms in the salivary and gastrointestinal tract, cell adhesion, autoaggregation, coaggregation and antimicrobial activity against pathogen strains, were also evaluated as a way to research potential beneficial properties on the host (Armando et al., 2011).

Thus, in this chapter we describe the development and application of an *in vitro* methodology to evaluate the aflatoxin B_1 binding ability, gastrointestinal tolerance and potential beneficial properties of *Saccharomyces cerevisiae* strains, useful to select the more appropriated microorganism to be assayed in expensive, complicated but necessary *in vivo* studies.

2.1 Study of microorganism-aflatoxin B_1 interaction

To select accurately the most efficient microorganism to bind AFB_1, is very important so as to protect against aflatoxicosis by inclusion of microorganisms in the diet. Usually, the methodology assayed has been a selection of several candidates using a unique mycotoxin concentration (Haskard et al., 2001; Hernandez-Mendoza et al., 2009; Peltonen et al., 2001; Shetty & Jespersen, 2006). Table 1, developed in our laboratory, is a clear example of this methodology and its analysis shows that the efficiency of the microorganisms is strain dependent, so that toxin removal ranged from 13 to 42% for LAB strains and 16 to 40% for the yeast strains tested.

Microorganism	Source	% AFB_1 bound ± SD[a]
***Lactobacillus acidophilus* Po$_{22}$**	Poultry cecum	42.8 ± 1.7
***L. acidophilus* Po$_7$**	Poultry	34.6 ± 1.6
***L. acidophilus* 24**	Dairy	32.6 ± 2.0
***L. casei* 1**	Dairy	27.6 ± 1.5
***L. fermentum* 23**	Human	34.6 ± 3.2
***L. acidophilus* CRL 1014**	ATCC collection[b]	25.4 ± 1.7
***L. fermentum* subsp. *cellobiosus* 408**	Poultry	13.2 ± 9.8
***Saccharomyces cerevisiae* RC016**	Pig gut	15.8 ± 3.6
***S. cerevisiae* RC012**	Feedstuff	29.6 ± 2.4
***S. cerevisiae* RC008**	Feedstuff	20.6 ± 2.6
***S. cerevisiae* RC009**	Feedstuff	16.4 ± 1.2
***S. cerevisiae* 01**	Poultry faeces	28.6 ± 3.5
***S. cerevisiae* 03**	Poultry faeces	26.6 ± 2.9
***S. cerevisiae* 05**	Poultry faeces	33.4 ± 1.9
***S. cerevisiae* 08**	Poultry faeces	36.4 ± 2.7
***S. cerevisiae* CECT 1891**	STCC collection[c]	40.0 ± 2.5

[a] The percentage of AFB_1 bound to cells was calculated as the difference between the total AFB_1 (5 µg ml^{-1}) and the amount of free AFB_1 (supernatant). Values are means ± standard deviations for duplicate samples.
[b]ATCC, American Type Culture Collection, Manassas, VA, USA.
[c] STCC, Spanish Type Culture Collection, University of Valencia, Valencia, Spain.

Table 1. Percentage of AFB_1 bound to cells upon exposure to viable microorganisms Bacteria, 2 x 10^8 CFU ml^{-1} and yeast 1 x 10^7 CFU ml^{-1} were incubated with 1 ml of AFB_1 (5 µg ml^{-1}) in PBS for 30 min at 37°C. The microorganisms were then pelleted by centrifugation, and the supernatant was collected for free AFB_1 analysis by HPLC according to Bueno et al. (2007).

The same experiment, but using three different concentrations of aflatoxin B_1, shows that the microorganism assayed to one concentration could not be the most efficient when the latter is changed (Table 2 and 3). Thus when AFB_1 concentration was 50 ng ml^{-1}, *S. cerevisiae* RC 016 was the most effective strain, but *S. cerevisiae* 08 and *S. cerevisiae* CECT 1891 were the best when AFB_1 concentration was increased at 100 ng ml^{-1}, and with 500 ng ml^{-1} 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 their concentrations were 50 and 100 ng ml^{-1}; however *S. cerevisiae* 01 was more effective at 500 ng ml^{-1} (Table 2). Lactic acid bacteria strains also showed the same behaviour, because *L. rhamnosus* I, *L. acidophilus* 24 and *L. casei* subsp. *rhamnosus* were the best at 50, 100 and 500 ng ml^{-1} respectively (Table 3).

Strains	AFB_1 concentration (ng ml^{-1})					
	50		100		500	
	AFB_1 binding					
	(ng ml^{-1})	%	(ng ml^{-1})	%	(ng ml^{-1})	%
***S. cerevisiae* CECT 1891**	10.0 ± 4.3	20.0	**57.6 ± 8.6**	**57.6**	255.0 ± 32.1	51.0
***S. cerevisiae* RC 008**	33.8 ± 0.1	67.6	45.6 ± 7.1	45.6	197.9 ± 24.1	38.2
***S. cerevisiae* RC 012**	15.3 ± 1.6	29.6	21.5 ± 3.1	21.5	103.7 ± 9.4	20.2
***S. cerevisiae* RC 009**	8.4 ± 0.8	16.8	21.5 ± 0.8	21.5	159.3 ± 1.2	31.8
***S. cerevisiae* RC 016**	**41.6 ± 1.9**	**82.0**	49.1 ± 1.4	49.1	**328.8 ± 5.2**	**65.7**
***S. cerevisiae* 01**	19.3 ± 1.2	38.6	31.7 ± 1.2	31.7	164.0 ± 6.9	32.8
***S. cerevisiae* 03**	23.3 ± 2.9	46.6	34.5 ± 1.8	34.5	128.7 ± 5.8	25.7
***S. cerevisiae* 05**	16.7 ± 1.2	33.4	24.0 ± 1.7	24.0	92.0 ± 6.9	18.4
***S. cerevisiae* 08**	23.2 ± 2.8	46.4	**58.9 ± 2.1**	**58.9**	187.0 ± 18.2	37.4

Cells (10^7 CFU ml^{-1}) were suspended in PBS in the presence of AFB_1 at the indicated concentration and incubated as described in Table 1. AFB_1 analysis by HPLC was performed according to Trucksess et al. (1994). Data are means ± standard deviations from three experiments in duplicate.

Table 2. Aflatoxin B_1 remotion by *Saccharomyces cerevisiae* strains at three different mycotoxin concentrations

Strains	AFB_1 concentration (ng ml^{-1})					
	50		100		500	
	AFB_1 binding					
	(ng ml^{-1})	%	(ng ml^{-1})	%	(ng ml^{-1})	%
Lactobacillus casei* subsp. *rhamnosus	18.0 ± 3.4	36.0	56.3 ± 5.4	56.3	**338.0 ± 7.2**	**67.6**
***L. rhamnosus* I**	**35.8 ± 1.3**	**71.6**	61.8 ± 3.1	61.8	254.7 ± 4.2	50.9
***L. fermentum* 23**	22.3 ± 1.2	44.6	41.0 ± 2.8	41.0	225.3 ± 10.8	45.1
***L. acidophilus* 24**	26.3 ± 1.9	52.6	**82.5 ± 3.2**	**82.5**	254.0 ± 25.9	50.8
***L. casei* 1**	13.8 ± 0.9	27.6	27.5 ± 1.3	27.5	59.0 ± 1.7	11.8

Cells (5×10^8 CFU ml^{-1}). AFB_1 binding to cells was performed as described in Table 2. Data are means ± standard deviations from three experiments in duplicate.

Table 3. Aflatoxin B_1 remotion by lactic acid bacteria strains at three different mycotoxin concentrations

Therefore, as AFB_1 concentration is highly variable in foods and feeds, the methods of selection using a unique aflatoxin B_1 concentration may lead to erroneous results. Another

very important condition to consider is how to quantify the concentration of employed microorganisms, CFU ml^{-1} or total cells ml^{-1}, where to evaluate whether viable and nonviable cells remove AFB_1 with the same efficiency becomes necessary.
Thus, the solution to these problems will be to know the mechanism involved in cell-aflatoxin interaction, and probably will also allow us to find the microorganism able to protect against aflatoxicosis in a wide range of mycotoxin concentration. In this sense, one of the objectives of the present study was to develop a theoretical model able to explain the binding of AFB_1 by LAB and *Saccharomyces cerevisiae* strains. With this purpose we studied as influences on the process involved the following conditions: microorganism-AFB_1 time contact, aflatoxin B_1 and microorganism concentration, cell viability, release of AFB_1 bound by cells and importance of the microorganism cell wall.

2.1.1 Microorganism-AFB_1 time contact

Table 4 summarizes some representative results (three LAB and three yeast strains) of the studies done in our laboratory when contact time between AFB_1 and the microorganisms were tested. By varying the incubation time, no significant difference in the amount of AFB_1 removed for LAB and yeast strains were observed. Furthermore, the process was fast, since in 1 minute the microorganism was able to bind the same amount of mycotoxin as in 6 h.

Time (min)	**AFB_1 Binding** ($ng\ ml^{-1}$)					
	***S. cerevisiae* 08**	***S. cerevisiae* RC016**	***S. cerevisiae* CECT 1891**	***L. fermentum* subsp. *cellobiosus* 408**	***L. casei* 1**	***L. acidophilus* P_{22}**
1	182.8 ± 18.2	318.5 ± 5.2	258.0 ± 14.2	61.5 ± 4.3	57.5 ± 2.2	89.4 ± 4.2
5	194.0 ± 12.6	312.8 ± 6.7	245.5 ± 10.3	64.3 ± 5.1	54.3 ± 3.1	91.8 ± 3.3
60	178.9 ± 15.7	332.0 ± 10.2	252.7 ± 15.6	62.8 ± 4.6	59.0 ± 4.2	88.9 ± 5.1
360	193.2 ± 14.6	326.6 ± 12.1	267.2 ± 12.6	63.8 ± 3.5	56.8 ± 2.9	92.3 ± 4.7

Table 4. Effect of incubation time on aflatoxin B_1 binding by viable cells of yeasts and LAB AFB_1 concentration: 0.5 μg ml^{-1}. Cells number: yeasts 1 x 10^7 CFU ml^{-1}; LAB 5 x 10^8 CFU ml^{-1}. AFB_1 binding was performed as described in Table 1, except that the incubation time varied as indicated in column 1. Data are means ± standard deviations from three experiments in duplicate. There is not significant differences ($P<0.05$) in the means values of each column.

These results are in agreement with other authors (El-Nezami et al., 1998; Peltonen et al., 2001) who have found no significant differences in AFB_1 removal by *E. coli*, *Propionibacterium* and several LAB strains after 72 h of incubation with the toxin. As for yeast, our results are consistent with those reported by Shetty et al. (2007), who have not observed differences between 0.5 and 12 h of time contact.

If the process needs so little time (one minute), it could suggest that neither the entrance of AFB_1 into cell nor its metabolic conversion is necessary, therefore microorganism cell wall components may be involved in aflatoxin B_1 remotion, as was suggested by various authors (Haskard et al., 2001; Karaman et al., 2005; Lahtinen et al., 2004; Raju & Devegowda, 2000).

2.1.2 Mycotoxin and microorganisms concentration

Effects of different AFB_1 concentration on toxin removal by LAB and yeast strains are shown in figure 1. Regardless of the studied strain, mycotoxin binding was dependent of its solution concentration and was always lineal at low values of AFB_1 and showed the transition to a plateau with higher toxin concentrations. The amount of toxin removed increased with increasing AFB_1 concentration, but the percentage removed decreased with increasing toxin concentration, because the saturation started. This behaviour strongly suggests that the microorganisms have a limited number of sites to bind AFB_1 either as free or occupied sites.

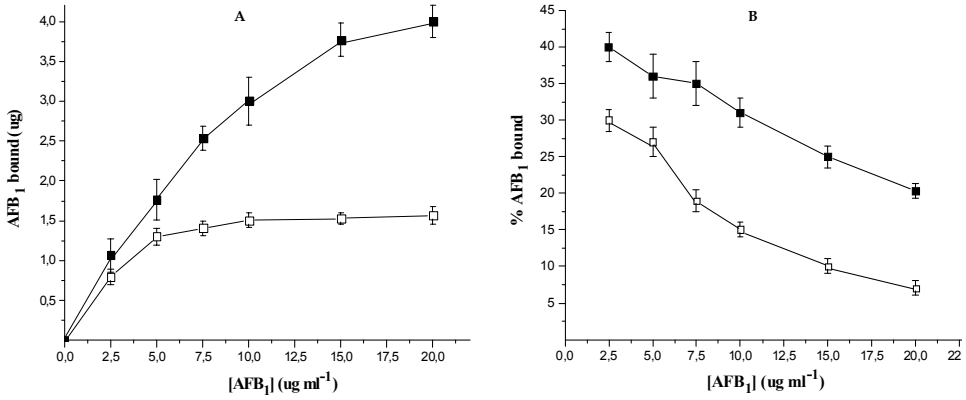
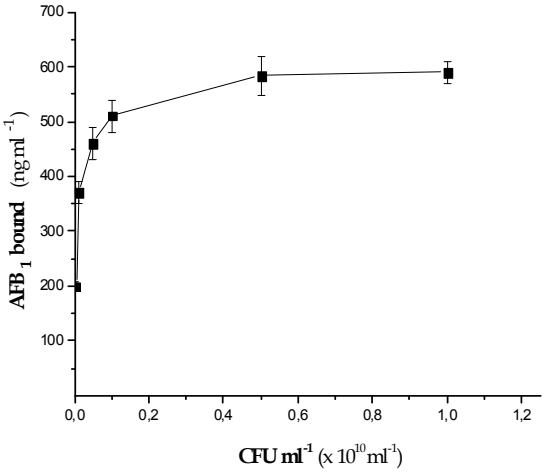


Fig. 1. (A) Adsorption isotherms of AFB_1 by *Lactobacillus acidophilus* 24 (□) and *Saccharomyces cerevisiae* 01 (■). Aliquots of 1 ml of cells (3 x 10^8 CFU ml^{-1}) for *L. acidophilus* 24 and (1 x 10^7 CFU ml^{-1}) for *S. cerevisiae* 01, were suspended in PBS in the presence of AFB_1 at the following concentrations: 2.5; 5.0; 7.5; 10.0; 15.0 and 20.0 µg ml^{-1}. AFB_1 binding to cells was performed as described in Table 1. (B) AFB_1 binding expressed as a percentage of the amount of mycotoxin present in the medium. Data are means from triplicate experiments.

Lee et al. (2003) refer to AFB_1 binding as a process of very high-affinity, linear relation with the toxin concentration used, and therefore, the amount of AFB_1 bound should be "limitless"; in other words they conclude that the bacterial surface does not have a defined number of binding sites. Our results do not support this idea. An important difference could be the number of microorganisms used in the experiments (10^{10} for Lee et al. and 10^8 for us), including more than a hundred times higher than ours for similar concentrations of AFB_1, so that the saturation phenomenon could not be observed.

When a growing number of microorganisms were suspended in PBS in the presence of a fixed AFB_1 concentration, we observed that the increase in bacterial or yeast concentration also reported an increase in AFB_1 binding, but it was never sufficient to bind all toxins present in the medium. Figure 2 shows the results with *Lactobacillus casei* subsp. *rhamnosus* which are similar to those obtained with all LAB and yeast strains we analysed.

Other authors, including El-Nezami et al. (1998), have also reported that removal of the toxin is cell number dependent and the bacterial increase was never sufficient to bind more than 90% of the toxin present in the environment. These results suggest that the process reached equilibrium between bound toxins (occupied sites) and unbound toxins (free sites) and therefore a reversible process could be involved in AFB_1 decontamination.



Cells, at the concentration indicated, were suspended in PBS in the presence of AFB_1 at a concentration of 750 ng ml^{-1} and incubated for 30 min at 37°C. AFB_1 binding to cells was performed as described in Table 2. Data are means ± standard deviations for triplicate samples.

Fig. 2. Effect of bacterial concentration on AFB_1 removal by *Lactobacillus casei* subsp. *rhamnous*

2.1.3 Cell viability

To further study the mechanism involved in AFB_1 removing, we examined whether cell viability affects the AFB_1 binding. Results in Table 5 did not show significant differences in remotion of the toxin by viable and nonviable cells (obtained by heat treatment).

Our results are similar to those obtained by El-Nezami et al. (1998) and some of the tested strains by Haskard et al. (2001), but different from other authors as Lee et al. (2003) and Shetty et al. (2007) who reported that heat treatment of cells enhanced their binding abilities among 20-50%. However, this effect was not observed in our study with none of the microorganisms tested (seven LAB and nine yeast strains, nine of them including in table 5). Therefore, the fact that non viable and viable microorganisms are able to eliminate AFB_1 with similar efficiency, suggests that the process involved does not require metabolic conversion of the toxin by cells and on the other hand, that the total microorganism number (cells ml^{-1}) should be employed in the estimation of cellular concentration, instead of CFU ml^{-1}. Additionally, these results are indicating that the inclusion of viable or nonviable microorganisms in the diet of animals would be equally effective against aflatoxicosis. This is very important because the possibility of using nonviable cells decreases the risks of their inclusion in the diet. Although the studies to ensure that these microorganisms are innocuous for animal health are not easy they become highly necessary.

Strains	AFB_1 binding (ng ml^{-1})	
	Viable	Nonviable
***L. fermentum* 23**	41.8 ± 3.6	45.0 ± 1.1
***L. acidophilus* 24**	83.4 ± 3.5	78.7 ± 2.9
L. casei* subsp. *rhamnosus	54.7 ± 6.5	56.3 ± 10.5
***L. rhamnosus* I**	61.8 ± 3.1	63.0 ± 2.1
L. paracasei* subsp. *paracasei	72.4 ± 5.5	69.5 ± 4.5
***L. casei* 1**	27.5 ± 3.8	23.5 ± 1.1
***S. cerevisiae* CECT 1891**	55.2 ± 7.5	53.0 ± 5.4
***S. cerevisiae* 08**	58.9 ± 2.1	55.8 ± 2.3
***S. cerevisiae* RC016**	49.1 ± 1.4	52.4 ± 1.7

Cells (10^7-10^8 cell ml^{-1}) were suspended in PBS in the presence of AFB_1 at a concentration of 100 ng ml^{-1} and incubated for 30 min at 37°C. AFB_1 binding to cells was performed as described in Table 2. Non viable cells were obtained by heat treatment (autoclaving for 20 min at 121°C) from the same sample of viable cells. Data are means ± standard deviations from three experiments in duplicate. There is not significant differences ($P<0.05$) in the means values of each row.

Table 5. Aflatoxin B_1 binding upon exposure to viable and nonviable cells

2.1.4 Aflatoxin B_1 released

The stability of the complex aflatoxin B_1-microorganism was studied by the application of repeated washings with PBS buffer (aqueous solvent) or acetonitrile (organic solvent in which AFB_1 is very soluble) of the cellular pellets that previously bound the mycotoxin, additionally the effect of the variation in the washing time was included.

Table 6 shows some representative results from all LAB and a yeast strains assayed in our laboratory. After five washings with PBS or acetonitrile, the AFB_1 released was ≥ 90% for the organic solvent treatment and close to 50% for the PBS treatment; on the other hand, the washing time did not change the release percentages when it varied from 1 to 60 minutes.

These results suggest that the process involved is fast and reversible. Moreover they confirm that metabolic conversion of the toxin by cells did not take place, because the aflatoxin B_1 was released in the same chemical form from microorganisms.

Reversibility of the process has previously been reported by other authors, for example, twelve LAB strains in both viable and non viable forms, tested by Haskard et al. (2001) exhibited reversible binding of AFB_1 after five washings too. Moreover, they also noted that the release of bound toxin was dependent on the washing solution employed, because only 6% to 11% of the bound AFB_1 was released using water, but when the complex was suspended in methanol, acetonitrile, chloroform, or benzene 83% to 99% of the bound AFB_1 was released. Similar findings have been reported by Peltonen et al. (2001) and Hernandez-Mendoza et al. (2009), the latter authors showed that employing PBS around 20-30% of AFB_1 bound was released and suggested that the aflatoxin B_1 is attached to the bacteria by weak, non covalent interactions that could be at least partially reversible.

Time (min)	AFB_1 released (%)							
	PBS				Acetonitrile			
	L. acidophilus P_{22}	*L. fermentum* subsp. *cellobiosus* 408	*L. casei* 1	*S. cerevisiae* CECT 1891	*L. acidophilus* P_{22}	*L. fermentum* subsp. *cellobiosus* 408	*L. casei* 1	*S. cerevisiae* CECT 1891
1	57.9 ±7.0	50.4 ± 3.5	42.3 ± 1.8	40.8 ± 2.1	98.3 ± 9.8	95.7 ± 5.6	94.4 ± 4.3	90.6 ± 4.1
10	58.7 ± 8.8	51.9 ± 2.7	43.8 ± 2.1	39.7 ± 1.2	97.0 ± 4.5	89.6 ± 8.9	91.8 ± 4.8	92.4 ± 3.9
60	61.0 ± 3.5	53.8 ± 3.9	44.1 ± 3.4	41.2 ± 1.5	98.4 ± 5.9	95.4 ± 5.8	95.9 ± 4.8	94.1 ± 4.2

For the aflatoxin release assay, cells (10^7-10^8 cells ml^{-1}) were incubated with AFB_1 at a concentration of 0.5 µg ml^{-1} for 30 min at 37°C and then centrifuged. Pelleted cells with bound AFB_1 were suspended in 1 ml of PBS or acetonitrile and incubated at the indicated times at 37°C with shaking. After that the microorganisms were pelleted by centrifugation, the supernatant containing the released AFB_1 was collected by HPLC analysis as was performed in Table 2. This process was repeated five times. The AFB_1 released (total from five washing) was expressed as a percentage of the total AFB_1 bound. Data are means ± standard deviations from triplicate experiments.

Table 6. Effects of different solvents and washing time on AFB_1 released by microorganisms

2.1.5 Importance of the microorganism cell wall

Although bibliographic data suggest that structural components of the cell wall of yeasts and LAB, are responsible in AFB_1-microorganism interaction (Karaman et al., 2005; Lahtinen et al., 2004), we designed an experiment to evaluate if yeast without cell wall (spheroplasts) are able to remove AFB_1, in order to confirm this assertion.

Sample*	AFB_1 binding			
	0.25 µg ml^{-1}#		2.5 µg ml^{-1}#	
	(µg ml^{-1})	(%)	(µg ml^{-1})	(%)
Cells control	0.14 ± 0.01[a]	56.0	1.25 ± 0.10[a]	50.0
Spheroplasts	0.02 ± 0.01[b]	8.0	0.05 ± 0.01[b]	2.0
Supernatant of spheroplasts	0.02 ± 0.00[b]	8.0	0.08 ± 0.01[c]	3.2

*Cells and spheroplasts: 10^7 ml^{-1}. #AFB_1 concentration in liquid media. The samples were suspended in 1 ml PBS in the presence of AFB_1 at a concentration of 0.25 µg ml^{-1} or 2.5 µg ml^{-1} and incubated for 30 min at 37°C. AFB_1 binding was performed as described in Table 2. Data are means ± standard deviations from triplicate experiments. Means with different letters in the same column differ significantly ($P<0.05$).

Table 7. Aflatoxin B_1 binding by cells, spheroplasts and supernantant of spheroplasts of *Saccharomyces cerevisiae* CECT 1891

The spheroplasts were obtained by the treatment with Zymolyase containing a protease activity with affinity for mannoproteins, and a β1,3 glucanase activity. The actions of both enzymatic activities were required to lyses yeast cell wall (Ovalle et al., 1998) and allowed us to obtain spheroplasts and the released products of the enzymatic breakdown, separately.

Thus, to determine the AFB_1 binding with three different samples: i) whole cells of *S. cerevisiae* CECT 1891 (cells control), ii) spheroplasts from cell control and iii) a concentrate of the supernatant from spheroplasts corresponding to 10^7 cells, was possible.
As Table 7 shows, neither spheroplasts nor its supernatant were able to remove AFB_1 from liquid medium, since very low uptake rates not even changed when the concentration of aflatoxin B_1 in the medium was increased 10 times, suggesting that these binds were nonspecific.
These results confirm that the compounds involved in AFB_1 binding to yeast, are components of the cell wall and that it must keep its structure in order to remove AFB_1 effectively. Similar results were reported by Hernandez-Mendoza et al. (2009), who demonstrated that the data obtained in binding assays with bacterial cell wall indicated that these purified fragments effectively bind AFB_1 as reported previously by Lahtinen et al. (2004). Furthermore, loss of the bacterial cell wall in response to treatments with enzymes showed a reduction in AFB_1 binding capacity relative to that of whole cells. These results demonstrate the importance of cell wall integrity in binding AFB_1 by LAB strains, and confirm the role of a cell wall-related physical phenomenon as opposed to a metabolic degradation reaction.

2.1.6 Mechanism proposed for the interaction between Aflatoxin B_1 with yeast and lactic acid bacteria strains

According to an integrated synthesis of the results reported above, it is clear that: (*i*) the removal and release of toxins is a fast and reversible process, (*ii*) this process does not involve AFB_1 chemical modification, (*iii*) the amount of AFB_1 removed is toxin- and cell concentration-dependent, (*iv*) the same result is obtained with viable and nonviable (heat-treated) cells and (*v*) the cell wall of the microorganism integrity is necessary for the "binding" mechanism between AFB_1 and the cells. Briefly, the process involved is, by nature, reversible and fast kinetic. Accordingly, this process can be analyzed as a **PHISICAL ADSORPTION** (physisorption), and probably the binding forces involved are a weak Van der Waals type, hydrogen bonds, or hydrophobic interaction.
Following this an adsorption phenomenon to the external microorganism surface to explain AFB_1 binding is proposed (Figure 3). 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. The shape of the isotherm shows linearity at low values of AFB_1 and then shows the transition to a plateau (Figure 4A). This type of isotherm can be described by the following equation:
$$\mathbf{Adsorption = M\,[AFB_1]_{eq}\,x\,K_{eq} / 1 + [AFB_1]_{eq}\,K_{eq}}$$ (Figure 3)
where **M** is the maximum number of adsorption sites per microorganism, and $\mathbf{K_{eq}}$ (expressed in liters per mole) is equivalent to the affinity (or cohesion force) of AFB_1 molecules for the adsorption sites. The linearized form of the isotherm is the double-reciprocal plot from the saturation curve ($\mathbf{1/adsorption = 1/[AFB_1]_{eq}\ 1/M\ K_{eq} + 1/M}$), as shown in Figure 4B. From the slope and interception of the resulting line, factors **M** and $\mathbf{K_{eq}}$ can be determined. The most efficient microorganism would be that having maximal M and K_{eq} values. Note that this physisorption model does not discriminate between viable and nonviable cells, i.e., cell concentrations should be determined by a hemocytometer instead of CFU per milliliter.

$$AFB_1 + S \rightleftharpoons S\text{-}AFB_1$$

$$K_{eq} = \frac{[S\text{-}AFB_1]}{[AFB_1]\,[S]} = \frac{Xso}{[AFB_1]\,Xsf}$$

$$Xso + Xsf = 1 \quad \text{and} \quad Ø = Xso$$

$$Xsf = 1 - Ø \quad \text{and} \quad K_{eq} = Ø / [AFB_1]\,(1 - Ø)$$

$$Ø = [AFB_1]\,K_{eq} / 1 + [AFB_1]\,K_{eq}$$

$$\text{Adsorption} = M \times Ø \quad \text{and} \quad \text{Adsorption} = \frac{M\,[AFB_1]\,K_{eq}}{1 + [AFB_1]\,K_{eq}}$$

$$1/\text{adsorption} = 1/[AFB_1]_{eq}\;\; 1/M\,K_{eq} + 1/M$$

Fig. 3. Theoretical model proposed to explain the adsorption process of AFB_1 by LAB and *S. cerevisiae*. The equations permit the calculation of the total binding sites per microorganism (M) and the equilibrium constant (K_{eq}) involved in the process. Sf is the amount of free sites in the surface cellular. So represents the occupied sites in the cell surface and is equivalent to S-AFB_1, determined as the AFB_1 bound to the cell. [AFB_1] is the AFB_1 concentration in the equilibrium condition of the system, determined as the free AFB_1 in the medium. Xso (Ø) is the molar fraction of occupied sites (mol So/mol total). Xsf (1 - Ø) is the molar fraction of free sites (mol Sf/mol total). Adsorption is the amount of molecules of AFB_1 bound per cell (M x Ø).

Figure 4A shows the saturation curve of *L. acidophilus* Po_{22} and *L. fermentum* subsp. *cellobiosus* 408 when the process is considered physisorption and indicates that the two strains have similar AFB_1 binding efficiency per bacterium, particularly for low concentrations of toxin. This result is different from the ones reported in Table 1 based only on viable bacteria (CFU ml^{-1}) and assayed with a unique mycotoxin concentration, since *L. acidophilus* Po_{22} was more efficient in AFB_1 binding (42.8%) than *L. fermentum* subsp. *cellobiosus* 408 (13.2%). However, we determined that in these assays Po_{22} strain had more dead cells than 408 strain, consequently Po_{22} had more total cells and showed higher percentage of AFB_1 binding. In terms of our proposed adsorption model, *L. fermentum* subsp. *cellobiosus* 408 has lower M values but higher K_{eq} values than *L. acidophilus* Po_{22} (Table 8), and these two factors balance to give toxin adsorption efficiencies of 9.37 and 6.25 x 10^{10} respectively.

According to adsorption model, a larger cell surface is associated with higher total sites per cell (M). To test this possibility, we measured AFB_1 adsorption in three yeast strains and the saturation curves are shown in Figure 5.

The adsorption of AFB_1 by *S. cerevisiae* RC016 (from pig gut), *S. cerevisiae* RC008 (from feed stuff) and *S. cerevisiae* CECT 1891 (from culture collection) was dependent on the toxin concentration in the medium, which is similar to the results showed in Figure 1 by

S. cerevisiae 01 and *L. acidophilus* 24. The data from Figure 5 were employed to construct the respective adsorption isotherms to obtain the M and K_{eq} values for these systems, and they are shown in Table 8. The M values were 25- to 1,000-fold higher for the yeast strains than for the bacteria, whereas K_{eq} values were similar, as differences never exceeded 3 times. Thus, the yeast strains respect to bacterial strains, showed a ~50-300-fold higher efficiency to AFB_1 removal from the medium, mainly for their high M values.

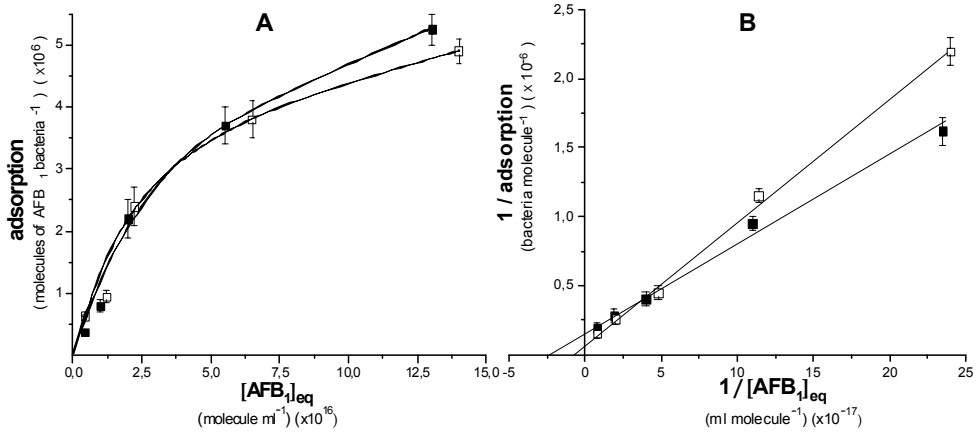
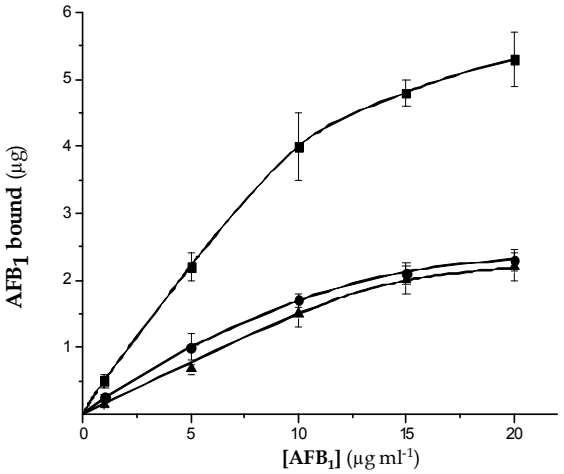


Fig. 4. Adsorption isotherms of AFB_1 by *L. fermentum* 408 (■) and *L. acidophilus* Po_{22} (□). Aliquots of 1 ml of cells (0.89 x 10^9 cells for *L. fermentum* 408; 1.20 x 10^9 cells for *L. acidophilus* Po_{22}) were suspended in PBS in the presence of AFB_1 at the following concentrations: 0.5; 1.0; 2.5; 5.0 and 10.0 µg ml^{-1}. Then, the bacteria were incubated for 30 min at 37°C and pelleted by centrifugation. The supernatant was collected for free AFB_1 analysis by HPLC according to Bueno et al. (2007). AFB_1 bound to cells was calculated as the difference between the total AFB_1 and the amount of free AFB_1. The adsorption was calculated as the ratio between the molecules of AFB_1 bound to cells and the amount of cells in the incubation medium. The $[AFB_1]_{eq}$ was equivalent to the free AFB_1. (A) Saturation curve. (B) Inverse plot of the same data as (A). Data are means from triplicate experiments.

Lee et al. (2003) described an adsorption process by three different bacteria strains, in viable and nonviable forms, as a function of AFB_1 concentration. For comparative purposes, we applied our theoretical model to the data of Lee et al. (Table 8, lines 3 through 5), and the M and K_{eq} values for their three LAB strains were calculated. *L. rhamnosus* LC- 705 had the most efficient AFB_1 removal. However, all three strains were less efficient than P_{22} and 408, mainly because they had smaller M values. Strain LGG-V of Lee et al. was 10-fold less efficient than 408, since its M value was 10-fold smaller as well (Table 8).

We proposed a theoretical model of adsorption applicable to microorganisms that bind AFB_1 and this model allows an estimation of the number of aflatoxin B_1 binding sites per microorganism (M), the system equilibrium constant (K_{eq}), and the efficiency of cells to remove AFB_1 from liquid medium (M x K_{eq}). We analyzed three systems: two *Lactobacillus* strains (*L. acidophilus* Po_{22} and *L. fermentum* subsp. *cellobiosus* 408) no tested before, three



Cells, at the concentration indicated, were suspended in 1 ml of PBS with different AFB_1 concentrations (1; 5; 10; 15; 20 µg ml^{-1}) and incubated for 30 min at 37°C. AFB_1 binding to cells was performed as described in Fig. 1. Data are means ± standard deviations for triplicate samples.

Fig. 5. AFB_1 binding upon exposure to *S. cerevisiae* CECT 1891 (1 x 10^7 cells ml^{-1}) (■), *S. cerevisiae* RC008 (4.8 x 10^7 cells ml^{-1}) (●), *S. cerevisiae* RC016 (2.5 x 10^7 cells ml^{-1}) (▲).

Strains	M (1x10^6 sites $cell^{-1}$)	K_{eq} (1x10^4 M^{-1})	Efficiency (1x10^{10})
***L. acidophilus* Po$_{22}$**	8.33	0.75	6.25
L. *fermentum* subsp. *cellobiosus* 408	6.25	1.50	9.37
LC705-NV	1.48	3.12	4.62
PJS-NV	1.00	2.80	2.80
LGG-V	0.64	1.40	0.89
***S. cerevisiae* RC016**	580.00	0.80	460.00
***S. cerevisiae* RC008**	200.00	2.20	440.00
***S. cerevisiae* CECT 1891**	1,000.00	3.12	3,120.00

Table 8. Total binding sites per microorganism (M), equilibrium constant (K_{eq}) and efficiency (M x K_{eq}) for different strains. M, K_{eq} and M x K_{eq} for various microorganisms were calculated by linear regression by the following equation: 1/adsorption = (1/[AFB_1]) x 1/M x K_{eq} + 1/M, as described in Fig. 3. The data for linear regression construction were extracted from Fig. 4A for *L. acidophilus* Po_{22} and L. *fermentum* subsp. *cellobiosus* 408 strains, from Fig. 5 for *S. cerevisiae* strains and from Fig. 1 from Lee et al. (2003) for LC705-NV (*Lactobacillus rhamnosus* strain, nonviable cells), PJS-NV (*Propionibacterium freudenreichii* subsp. *Shermanii* JS, nonviable cells), and LGG-V (*L. rhamnosus* GG, viable cells).

yeast strains (*S. cerevisiae* RC016, *S. cerevisiae* RC008 and *S. cerevisiae* CECT 1891) no studied before either, and LAB studied by another laboratory (Lee et al. 2003). The most efficient microorganism was *S. cerevisiae* CECT 1891, mainly because binds more AFB_1 per cell (Table 8). As we mentioned before, in AFB_1 binding to the yeast, the main components involved are cell wall glucomannans (Karaman et al., 2005), whereas cell wall peptidoglycans are responsible for AFB_1 removal by LAB (Lahtinen et al., 2004). Unexpectedly, bacteria and

yeast had a K_{eq} value within the same order of magnitude, varying from 0.75 to 3.12 x 10^4 M^{-1}; in addition, *S. cerevisiae* CECT 1891 and LC705-NV had the same K_{eq} (Table 8). High efficiency resulted from high M value rather than from any variation in the K_{eq} value. These observations suggest that binding efficiency is a more quantitative phenomenon (large surface area) than a qualitative phenomenon (chemical structure involved in the interaction), and in this sense the analysis of the glucomannan and peptidoglycan chemical structures did not indicate major differences in the capacity to produce hydrophobic interactions or hydrogen bonds, which are presumably involved in the binding. The main application of the proposed model is its usefulness for the selection of the most efficient microorganism to remove AFB_1. *Sacharomyces cerevisiae* CECT 1891 was clearly the most efficient microorganism in the present study because it had high values of M and K_{eq}. When the election is between microorganisms with similar efficiencies (M x K_{eq})—for example, *L. acidophilus* P_{22} and LC705-NV or *S. cerevisiae* RC016 and *S. cerevisiae* RC008 (see Table 8)—the model suggests that the election should be the cell with the high K_{eq} factor (LC705- NV and *S. cerevisiae* RC008, respectively). In fact, when a microorganism-AFB_1 complex is exposed to consecutive washings, for example during gastrointestinal transit, the microorganism with the high value of K_{eq} will release few toxins. This is consistent with the results of Lee et al. (2003), who determined the constants of AFB_1 release for LC705-NV, PJS-NV, and LGG-V, which showed an inverse correlation with the respective K_{eq} values calculated by us (Table 8) and therefore the greatest constant of release of minor K_{eq} factors. Our results for *L. acidophilus* P_{22} and *S. cerevisiae* CECT 1891 also show the same correlation, i.e., that *L. acidophilus* P_{22} has a greater constant of release of minor K_{eq} factors than *S. cerevisiae* CECT 1891 (Table 8) and releases a greater percentage of AFB_1 than yeast strain does, when both are exposed to washings (Table 6). Therefore, it is clear that the microorganism with the greater K_{eq} factor has less toxin releases and this fact will be very important during passage through the gastrointestinal tract, where mainly aflatoxins are absorbed (Gratz et al., 2004).

When we analyzed the effect of mycotoxin and microorganism concentration, we remark the differences with the results obtained by Lee et al. (2003) who did not report saturability during the AFB_1 binding process, and we suggest that the high cell concentration employed by these authors was responsible of this non-saturation. For the same reason, our experiments with yeast strains were made with concentrations of 10^7 cells per ml, 100 times less than for bacteria; otherwise, the saturation was hidden.

Another important use of this adsorption model is its capacity to explain probable changes in the adsorption efficiency of the microorganisms (M x K_{eq}) after physical, chemical, or genetic treatments on the microorganisms. In fact, we can determine whether such changes are induced by modifications of M, K_{eq}, or both. This knowledge will help us to select the most efficient microorganisms for protection against AFB_1.

Although the results described come from in vitro experiments, some preliminary experiments in our laboratory (data not shown) suggest that the addition of *S. cerevisiae* CECT 1891 to poultry diet would help to prevent acute aflatoxicosis, chronic aflatoxicosis, or both.

In summary, when experiments *in vitro* made with a definite microorganism concentration and variable AFB_1 concentration show saturability, the adsorption model will be applicable and will allow a determination of M and K_{eq} values, both of which are useful when selecting the most efficient microorganism to remove AFB_1 in a wide range of mycotoxin concentration.

2.2 Evaluation of the gastrointestinal tolerance and anti-pathogenic bacterial influence of *Saccharomyces cerevisiae* strains

An interesting alternative to protect against aflatoxicosis, after the selection of the most efficient microorganism in AFB_1 remotion is to research its potential beneficial properties on

the host. While our proposed model above includes the possibility of using dead microorganisms and with the same aflatoxin B_1 binding efficiency, the inclusion in the diet of viable cells would add other beneficial effects to the animal we want to protect.
So, if the aim is to use living microorganisms, the first study to perform is to evaluate their tolerance to the gastrointestinal conditions, because acid in the stomach and bile salts in the gut are the first biological barriers to be overcome after ingestion (Gueimonde & Salminen, 2006).
In recent years, much attention has been paid to the design of functional foods that contain probiotic microbial strains responsible for health benefits in the host (Kumura et al., 2004). Several authors have demonstrated the safety of *Saccharomyces cerevisiae* for nutritional and pharmaceutical use in animal feed in the European Economic Community and in Japan (Nitta & Kobayashi, 1999). Moreover, the Food and Drug Administration has given level of insurance or GRAS (generally regarded as safe) microorganism degree to *Saccharomyces cerevisiae* strains (http://www.fda.gov/Food/FoodIngredientsPackaging/ucm078956.htm).
Microorganisms capable of withstanding the gastrointestinal transit (≥70% survival), have drawn attention to their possible use as probiotics (Lourens-Hattingh & Viljoen, 2001), and in this sense these microorganisms will be considered for further studies in order to evaluate their potential beneficial properties.
We employed this strategy to study four *Saccharomyces cerevisiae* strains isolated from pig environment, previously analyzed for their AFB_1 binding ability (Table 1 and 2), including assays for: *i*) gastrointestinal tolerance, *ii*) auto-aggregation, *iii*) cellular adhesion, *iv*) co-aggregation and *v*) antagonism to pig pathogens (Armando et al., 2011)

2.2.1 Selection of *Saccharomyces cerevisiae* strains for their tolerance to gastrointestinal conditions

The effect of simulated GI transit on viability of *S. cerevisiae* strains is presented in Table 9. All the yeast strains were able to survive under gastrointestinal conditions. In general, the strains retained viability through salivary (M2) conditions. *Saccharomyces cerevisiae* RC009 strain showed an increase in viability during transit to gastric (M3) and intestinal (M4) conditions; whereas *S. cerevisiae* RC008 strain did not increase the cell density but maintained the number of viable cells throughout the gastrointestinal transit. *Saccharomyces cerevisiae* RC012 showed a small decrease in viability after passage intestinal conditions (M4). In contrast, the RC016 strain was able to grow at these conditions.

Source	*S. cerevisiae* Strains	Viable count (log_{10} CFU ml^{-1}) during simulated GI transit tolerance			
		M_1	M_2	M_3	M_4
Feedstuff	**RC008**	6.4 ± 0.40 ^{a}	6.7 ± 0.17 ^{a}	6.5 ± 0.06 ^{a}	6.8 ± 0.11 ^{a}
Feedstuff	**RC009**	6.7 ± 0.23 ^{b}	6.4 ± 0.11 ^{b}	7.2 ± 0.17 ^{a}	7.5 ± 0.21 ^{a}
Feedstuff	**RC012**	6.4 ± 0.26 ^{b}	6.9 ± 0.15 ^{b}	6.4 ± 0.21 ^{b}	6.1 ± 0.26 ^{c}
Pig gut	**RC016**	7.3 ± 0.17 ^{a}	7.1 ± 0.15 ^{b}	5.8 ± 0.17 ^{c}	7.5 ± 0.06 ^{a}

Results are shown as mean ± SD (standard deviation). M_1: cell counts prior to assaying the GI transit tolerance. M_2: salivary conditions tolerance assay. M_3: gastric conditions tolerance assay. M_4: intestinal conditions tolerance assay. Values with the same letter are not significantly different according to Fisher's protected LSD test ($p<0.0001$). Statistical analysis compared means obtained from each yeast strain separately.

Table 9. Effect of simulated gastrointestinal (GI) transit on viability of *Saccharomyces cerevisiae* strains

The ability to survive gastrointestinal simulated conditions is an absolute need of probiotic microorganisms, and it is generally included among the criteria used to select potential probiotic strains (Morelli, 2000). In this work, all the assayed strains were able not only to resist gastrointestinal passage but also to grow under these conditions. Other authors have reported the same results with *S. cerevisiae* strains isolated from infant faeces and feta cheese (Psomas et al., 2001) and with *S. cerevisiae* var *boulardii* strain isolated from food (van der Aa Kuhle et al., 2005). However, the use of bacteria has demonstrated a very low recovery after being subjected to these gastrointestinal *in vitro* conditions (Gusils et al., 2002). Lin et al. (2007) reported a 2 or 3 log decrease in *Lactobacillus fermentum* strains isolated from swine and poultry. In agreement with our results, Pennacchia et al. *(*2008) reported that more than 50% of *S. cerevisiae* strains exposed to simulated passage through the human GI tract, showed 70% survival.

2.2.2 Auto-aggregation assay

Table 10 shows the auto-aggregation ability of the tested *S. cerevisiae* strains. Results showed that RC008, RC009 and RC016 strains exhibited a strong auto-aggregation score while RC012 strain exhibited weak auto-aggregation. This ability, or formation of multicellular clumps between microorganisms of the same strain, is a measure of the adhesion ability to epithelial cells and could be related to biofilm formation. The percentages of auto-aggregation obtained ranged from 85.3 to 97.9%, indicating that all strains showed an auto-aggregative phenotype.

***S. cerevisiae* Strains**	**OD_{600} (t_0)[1]**	**OD_{600} (t_1)[2]**	**[1- (OD_{600} t_1/ OD_{600} t_0)] x 100 %**	**Aggregation Score[3]**
RC008	1.144	0.088	92.3	++
RC009	1.173	0.025	97.9	++
RC012	0.974	0.144	85.3	+
RC016	1.099	0.079	92.9	++

[1]Cells were harvested by centrifugation and suspended in PBS to optical density ~1 (O.D.) units at 600 nm. [2]Optical density of the upper suspension (O.D.) units at 600 nm after incubation at 37ºC for 2 h. [3] (++) strong; (+) weak; (-) negative

Table 10. Auto-aggregation ability of *Saccharomyces cerevisiae* strains

2.2.3 Adhesion assay

The number of yeast cells adhesive to 100 Vero cells at different inoculums sizes of *S. cerevisiae* is shown in Table 11.

Results indicate that the adhesion ability of yeasts varied among strains according to the density of yeast cells. At 10^6 cells ml^{-1} the strains showed different adhesion ability: some of them had high adhesion capability while in others it was low. At 10^7 cells ml^{-1} all strains showed a similar strong adhesive ability to Vero cells. Adhesion ability of probiotcs to intestinal mucus and to enterocytes is an important prerequisite for transient colonization of the host intestinal tract. Adhesion to the mucosa is also considered to be an important factor for modulation of the immune system and for antagonistic activity against enteropathogens

(Ouwehand et al., 2002). Some authors reported a strong relationship between auto-aggregation and adhesiveness (Del Re et al., 2000), in line with the results obtained by us.

S. cerevisiae Strains	Cells ml^{-1}	Yeast cell number / 100 Vero cells	
		Mean ± SD[1]	LSD test[2]
RC008	10^6	18.5 ± 0.16	g
	10^7	154.0 ± 0.09	b
RC009	10^6	107.3 ± 0.09	d
	10^7	178.2 ± 0.02	a
RC012	10^6	109.5 ± 0.13	c
	10^7	154.8 ± 0.26	b
RC016	10^6	48.9 ± 0.04	f
	10^7	105.9 ± 0.15	e

Twenty randomized microscopic fields per coverslip were counted. Each adhesion assay was conducted in duplicate with cells from three successive passages. Adhesion was expressed as a mean of yeast adhering per 100 Vero cells. Controls without *S. cerevisiae* strains were included. [1]Mean ± Standard Deviation (SD). [2]Values corresponding to the same letter are not significantly different according to Fisher's protected LSD test ($P<0.05$).

Table 11. Adhesion of *Saccharomyces cerevisiae* strains to Vero cells

2.2.4 Co-aggregation assay

Co-aggregation among *S. cerevisiae* and pathogens is shown in Table 12. The results showed that the capacity of yeast to bind to a microorganism varies according to yeast strain and the microorganism involved. Some yeast strains varied from non co-aggregation to good co-aggregation ability when they interact with *E. coli, Salmonella* sp. or *Enterobacter* sp.. *Saccharomyces cerevisiae* RC016 strain was able to co-aggregate with all the pathogens tested, while the rest of the strains only co-aggregate with one of the pathogenic bacteria.

S. cerevisiae Strains	Co-aggregation score[1]		
	E. coli	*Enterobacter sp.*	*Salmonella sp.*
RC008	0	2	0
RC009	0	0	1
RC012	0	0	1
RC016	1	1	1

Yeast suspension (1×10^7 CFU ml^{-1} in PBS) was mixed with each pathogen strain (1×10^7 CFU ml^{-1} in PBS). Suspension was mixed and incubated for 4 h at 37°C, under agitation at 200 rpm. Suspensions were observed by optic microscopy after Gram staining. [1]The score is based upon a scale described by Mastromarino et al. (2002), from 0 for no aggregation to 3 for maximum aggregation.

Table 12. Co-aggregation assay between *Saccharomyces cerevisiae* and pathogen strains

Co-aggregation and inhibition of pathogens are some of the most important beneficial health claims of probiotics. The co-aggregation ability of probiotic strains might enable them to form a barrier that prevents colonization by pathogenic bacteria (Collado et al., 2007).

2.2.5 Antagonism to pig pathogens

The antibacterial activity of yeast strains towards potential pathogenic bacteria was assayed (Table 13). Three of the tested *S. cerevisiae* were inhibitory against the pathogens studied. The RC008 and RC016 strains had the strongest antimicrobial activity; they were able to inhibit all the pathogens tested.

Most yeast strains are able to produce diffusible antimicrobial substances that inhibit the growth of pathogens. It was reported that certain *S. cerevisiae* strains secrete a protein toxin that kills not only sensitive strains of the same species but also other yeasts (Kitamoto et al., 1999). In our study, yeasts were able to inhibit pathogenic bacteria, but the nature of the antimicrobial substance is unknown.

***S. cerevisiae* Strains**	**Antimicrobial activity[1]**		
	Escherichia coli	***Salmonella enterica***	***Enterobacter cloacae***
RC008	**++**	**++**	**++**
RC009	**-**	**-**	**-**
RC012	**+**	**+**	**+**
RC016	**++**	**++**	**++**

[1] +: inhibition zone ≥ 3 mm and ≤ 9 mm;
++: inhibition zone ≥ 10 mm and ≤ 15 mm;
-: inhibition zone ≤ 3 mm.
A central streak of each yeast strain was performed on Petri dishes containing YPD agar and incubated for 48 h at 37°C. Ten millilitres (10 ml) of additional liquid YPD agar were added to each plate and each pathogenic strain was streaked (perpendicularly) across the same agar plate. After 24 h incubation, antagonistic effect was determined by the appearance of clear zones surrounding the junctions of the streak lines.

Table 13. *Saccharomyces cerevisiae* antimicrobial activity against pathogen strains

In summary, the results let us predict that *S. cerevisiae* RC016 and *S. cerevisiae* strain RC008 may be regarded as the most promising beneficial yeast candidate for functional feed product development because they both had good and similar AFB_1 remotion efficiency (Table 8), higher than *S. cerevisiae* RC009 and *S. cerevisiae* RC012 (data not shown) and the strongest capacity of pathogen inhibition. They were also able to survive under gastrointestinal conditions and adhere to intestinal cells. Other functional and technological tests should be performed for the validation of these strains as suitable probiotics for animals, and future studies should be conducted to evaluate the influence of *S. cerevisiae* strains on non-pathogenic bacteria. But evidently *S. cerevisiae* RC008 and *S. cerevisiae* RC016 are the best candidates, from the four yeast strains analyzed, for further studies *in vivo* designed to prevent aflatoxicosis.

3. Conclusions

As we have seen, the potential presence of aflatoxins in animal diet is unavoidable, therefore a protection against aflatoxicosis is necessary, and the inclusion in the diet of microorganisms able to remove AFB_1 is the most suitable alternative. Moreover, if the

microorganisms capable to avoid the aflatoxin absorption during its gastrointestinal transit have beneficial properties on the host, this way to prevent aflatoxicosis is highly promising. With the increasing interest in food safety throughout the world, the yeast and LAB cultures with high mycotoxin binding abilities and probiotic abilities are of immense value in reducing aflatoxin exposure.

The first section of this work presents *in vitro* experiments useful to the development of a model that considers the aflatoxin-microorganism interaction as a fast, reversible and strain specific process, concluding that it is a physical adsorption to the cell wall of the microorganism. The model is a tool for selecting the most efficient microorganism to remove AFB_1 in a wide range of mycotoxin concentration, since feed AFB_1 contamination is variable. Another important use of this model is its capacity to explain probable changes in the adsorption efficiency ($M \times K_{eq}$) of the microorganism after than physical, chemical or genetic treatments designed to obtain the ideal microorganism; i. e. if either the process equilibrium constant (K_{eq}) or the number of binding sites for AFB_1 in the surface microorganism (M) change, or both.

The application of the adsorption model to yeast and LAB strains showed that *Saccharomyces cerevisiae* CECT 1891 was the most efficient microorganism in AFB_1 remotion, but in general also showed that the yeasts had higher efficiencies than LAB, mainly because the M values were 20- to 1,000- fold higher for the yeasts than for the bacteria, whereas the K_{eq} values were similar.

The second part of this chapter shows the design of simple experiments *in vitro* to evaluate potential beneficial properties of the microorganisms under study, on the host. In this sense, the tolerance of gastrointestinal conditions, auto and co-aggregation, cell adhesion and antibacterial activity towards pathogenic bacteria, of four yeast strains from pig environmental, were assayed. The results showed that *Saccharomyces cerevisiae* RC016 and *Saccharomyces cerevisiae* RC008 were the best microorganisms, mainly because they showed high and similar AFB_1 remotion efficiency and the strongest antimicrobial activity against pathogen strains.

Thus, the combination of both strategies allows us to select, among all the microorganisms tested (LAB and yeasts), the best candidate for future *in vivo* studies. This selection is very important because, although *in vivo* studies are necessary, they are expensive, complicated and long lasting as well. So, in summary, this research represents an efficient *in vitro* strategy to select the correct microorganisms for future *in vivo* studies, useful to prevent aflatoxicosis in farm animals.

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