International Journal of Food Microbiology 156 (2012) 214–221

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Analysis of fumonisin B_1 removal by microorganisms in co-occurrence with aflatoxin B_1 and the nature of the binding process

Romina P. Pizzolitto [a,*], Mario A. Salvano [a], Ana M. Dalcero [b]

[a] Departamento de Biología Molecular, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 km 601, 5800 Río Cuarto, Córdoba, Argentina
[b] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36 km 601, 5800 Río Cuarto, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 1 November 2011
Received in revised form 24 February 2012
Accepted 22 March 2012
Available online 28 March 2012

Keywords:
Saccharomyces cerevisiae
Lactic acid bacteria
Fumonisin B_1
Aflatoxin B_1
Physical adsorption

ABSTRACT

The objectives of this investigation were to evaluate the ability of *Saccharomyces cerevisiae* CECT 1891 and *Lactobacillus acidophilus* 24 to remove fumonisin B_1 (FB_1) from liquid medium; to determine the nature of the mechanism involved in FB_1–microorganism interaction and to analyze whether the presence of aflatoxin B_1 (AFB_1) interferes with the removal of FB_1 and vice versa. The results obtained indicated that: (i) both microorganisms were able to remove FB_1 from liquid medium; (ii) the removal was a fast and reversible process; (iii) cell viability was not necessary; (iv) the amount of FB_1 removed was both toxin- and microorganism concentration-dependent; (v) the process did not involve chemical modification of FB_1 molecules; and (vi) cell wall structural integrity of the microorganisms was required for FB_1 removal. Consequently, we propose that the mechanism involved in the removal of FB_1 is a physical adsorption (physisorption) of the toxin molecule to cell wall components of the microorganisms. It is highly probable that FB_1 and AFB_1 co-occur in contaminated foods, since the fungal genera *Aspergillus* and *Fusarium* frequently occur simultaneously. Therefore, we analyzed whether the presence of AFB_1 interferes with the removal of FB_1 by the microorganisms previously evaluated, and vice versa. Studies of co-occurrence of both mycotoxins clearly showed that they did not compete for binding sites on the microorganism cell wall and the presence of one toxin did not modify the efficiency of the organism in the removal of the other mycotoxin. These findings may be useful for optimization of mycotoxin binding and provide an important contribution to research on microorganisms with ability to remove these secondary metabolites.

1. Introduction

Human diets contain a wide variety of natural carcinogens, such as mycotoxins, that are present in foods as a result of contaminated raw materials or they may be produced during the processing and/or storage of foods. Fumonisins are fungal secondary metabolites produced by species of *Fusarium*, mainly *Fusarium verticillioides* and *Fusarium proliferatum* (CAST, 2003; Keller et al., 1997; Krska et al., 2007). There are several identified fumonisins, but fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) are the most important and constitute up to 70% of the fumonisins found in naturally contaminated foods (Niderkorn et al., 2009). FB_1 and FB_2 are phytotoxic to corn (Lamprecht et al., 1994), cytotoxic to various mammalian cell lines (Abbas et al., 1993) and FB_1 is carcinogenic to rat liver and kidney (IARC, 2002). The occurrence of fumonisins in home-grown corn has been associated with an increased risk of esophageal cancer in humans (Shephard et al., 2000). FB_1 is the most toxic and is considered to be a possible human carcinogen and is classified as class 2B (IARC, 2002). This mycotoxin is the causal agent of two well described diseases in domestic animals: equine leukoencephalomalacia (Riley et al., 1997) and porcine pulmonary edema syndrome (Harrison et al., 1990). The mechanism of action appears to involve mainly disruption of sphingolipid biosynthesis by the inhibition of the enzyme sphingosine *N*-acetyltransferase (ceramide synthase) (Voss et al., 2007).

Considering the impact of fumonisins on health, the application of strategies to prevent their formation, as well as to eliminate, inactivate or reduce their presence in food products, is desirable. Most of the approaches have not been adopted due to high costs, loss of nutritional and sensory properties of the products, or practical difficulties involved in detoxification process (Firmin et al., 2011). Therefore, a promising alternative is the use of microorganisms as FB_1 sequestering agents. Inclusion of such microbes in the diet may reduce the toxic effects of mycotoxins on humans, as a FB_1–microorganism complex may decrease availability of the mycotoxin and consequently its absorption in the gastrointestinal tract (Gratz et al., 2007;

Abbreviations: FB_1, fumonisin B_1; FB_2, fumonisin B_2; AFB_1, aflatoxin B_1; PBS, phosphate-buffered saline; MW, molecular weight; LAB, lactic acid bacteria; Sa/So, sphinganine to sphingosine; MRS, De Man, Rogosa, Sharpe; YPD, Yeast Extract Peptone Dextrose; HPLC, high-performance liquid chromatography; OPA, o-phthaldialdehyde.

* Corresponding author. Tel.: +54 358 4676421; fax: +54 3584676232.
E-mail address: rpizzolitto@exa.unrc.edu.ar (R.P. Pizzolitto).

0168-1605/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2012.03.024

Hernandez-Mendoza et al., 2009). *Saccharomyces cerevisiae* and lactic acid bacteria (LAB) have been used as probiotics and potential mycotoxin decontaminating microorganisms because of their ability to bind these toxic metabolites. Nevertheless, despite several publications having reported *in vitro* binding by LAB and yeast strains of mycotoxins such as aflatoxin B_1 (AFB_1) (Bueno et al., 2007; Hernandez-Mendoza et al., 2009; Pizzolitto et al., 2011; Shetty and Jespersen, 2006), zearalenone and certain trichothecenes (El-Nezami et al., 2002a,b; Niderkorn et al., 2006, 2007) little is known about the mechanism involved in fumonisin removal. An understanding of the binding mechanism is required to allow the optimization and safe dietary application of this methodology. In addition, it is highly probable that FB_1 and AFB_1 co-occur in contaminated foods, since the fungal genera *Aspergillus* and *Fusarium* frequently occur simultaneously (Oliveira et al., 2006; Pietri et al., 2009; Rocha et al., 2009).

In previous studies we demonstrated that *S. cerevisiae* CECT 1891 and *Lactobacillus acidophilus* 24 showed high efficiency in AFB_1 removal from liquid media (Bueno et al., 2007; Pizzolitto et al., 2011). We were interested therefore, in identifying the nature of the mechanism involved (physical or chemical) in the FB_1 binding process, characterizing the interaction of selected strains in terms of binding conditions and dissociation of FB_1 and in addition, understanding if FB_1 removal is modified by the presence of AFB_1 and vice versa. To our knowledge, there is no information available that analyzes the binding of these mycotoxins in co-occurrence. The ability of microorganisms to bind AFB_1 and FB_1 could decrease the bioavailability of these compounds and limit their toxic effects on humans. In the present study, the removal of FB_1 and AFB_1 in co-occurrence from liquid medium by microorganisms was investigated, for the first time. The objectives of this investigation were: i) to evaluate the capacity of *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 to remove FB_1 from liquid medium; ii) to determine the nature of the mechanism involved in FB_1-microorganism interaction and iii) to analyze whether the presence of AFB_1 interferes with the removal of FB_1 and vice versa.

2. Materials and methods

2.1. Microorganisms and culture conditions

L. acidophilus 24 from the National University of Río Cuarto Culture Collection, was grown on De Man, Rogosa, Sharpe (MRS) broth under microaerophilic conditions for 24 h at 37 °C without shaking, using inoculum at 0.1% (v/v) from an overnight culture at 37 °C.

S. cerevisiae strain CECT 1891 from Spanish Type Culture Collection, University of Valencia, Valencia, Spain, was grown on Yeast Extract Peptone Dextrose (YPD) broth (0.5% yeast extract and 0.5% peptone, 4% glucose) in an orbital incubator at 150 rpm for 24 h at 25 °C. The inoculum of the yeast strain was prepared from an overnight culture in YPD broth at 25 °C. Then, 250 ml Erlenmeyer flasks containing 100 ml of YPD were inoculated with 1 ml of the respective inoculum. Cell suspension concentration was determined using a hemocytometer. Viability was confirmed by standard count methods using MRS or YPD agar.

2.2. Preparation of spheroplasts

A spheroplast is a cell from which the cell wall has been removed almost completely. The cell wall lysis assay was based on the method described by Ovalle et al. (1998) with some modifications. Briefly, *S. cerevisiae* CECT 1891 was grown in 100 ml of YPD broth at 25 °C with shaking until an optical density (OD) of 1 at 600 nm was achieved. Then, cells were centrifuged at 5000 *g* for 15 min and the pellet was washed with 0.022 M phosphate-buffered saline (PBS), pH 7.4. Cells were suspended in 10 ml of TSD buffer (0.1 M Tris sulfate pH 9.4, 0.01 M dithiothreitol), to obtain a solution with an OD 10 times greater, and incubated for 10 min at room temperature. This solution was then centrifuged at 4000 *g* for 5 min, the supernatant discarded and the pellet suspended with 3.3 ml of spheroplast A medium (0.022 M PBS, glucose 2% (w/v), 1 M sorbitol, 0.02 M Tris–HCl pH 7.5) with the final OD 30 times higher than the initial. Ten μl of this solution was diluted with 990 μl of deionized water, and the OD was measured at 600 nm and the value was recorded. Zymolyase (150 mg; AMS Biotechnology LTD), was then added to 3.3 ml of the cell suspension and incubated in a water bath without shaking at 37 °C. At 5-min intervals, 10 μl aliquots were taken and diluted with 990 μl of deionized water and their OD was measured at 600 nm and recorded. This process was carried out until the OD was 10 times lower than the initial. Then, the cell suspension (3.3 ml) was centrifuged at 1500 *g* for 5 min at room temperature. The pellet was washed once with spheroplast B medium (0.022 M PBS, glucose 2% (w/v), 1 M sorbitol) and after confirming under a microscope that the cells had been fully converted to spherical cells (spheroplasts) they were resuspended in 20 ml of 0.022 M PBS, pH 7.4 supplemented with 0.5 M sorbitol.

2.3. Fumonisin B_1 binding assay

FB_1 solid (Sigma, St. Louis, MO, USA) was suspended in acetonitrile:water (1:1, v/v) to obtain a FB_1 concentration of 1 mg/ml. Solutions of FB_1 (3.125; 6.25; 12.5; 15; 20; 25; 50 and 100 μg/ml) were prepared in PBS, pH 7.4.

To conduct mycotoxin binding assays, a volume of culture was centrifuged at 5000 *g* for 15 min at room temperature and the supernatant was removed. The cell concentration of the pellet was determined using a hemocytometer or plate count methods. The pellet was washed twice with PBS. The microbial pellet was then suspended in 1 ml of PBS containing FB_1, incubated for 30 min at 37 °C with shaking. At the end of the incubation period, tubes were centrifuged (5000 *g*, 10 min) and supernatants containing unbound FB_1 were collected and stored at −20 °C for high-performance liquid chromatography (HPLC) analysis (see below).

To study the effect of incubation time, cells and FB_1 (5 ml) were incubated for up to 270 min, and samples (1 ml) were collected at 1, 30, 60 and 270 min.

The effect of cell viability on FB_1 binding ability was carried out by heat-treatment of the cells (autoclaving for 20 min at 121 °C) before incubation with the toxin. Nonviable cells were monitored by plating in YPD or MRS medium.

Removal of FB_1 by spheroplasts was carried out as described above for whole cells, but the liquid medium (0.022 M PBS, pH 7.4) was supplemented with 0.5 M sorbitol, both for spheroplasts and control cells. The removal of FB_1 by the spheroplast supernatant was carried out with supernatant obtained from 10^7 spheroplasts/ml, which was also supplemented with 0.5 M sorbitol. Furthermore, after the incubation time, all samples were centrifuged at 25,000 *g* for 30 min, to obtain the supernatants containing the unbound FB_1. Positive (PBS + mycotoxin) and negative (PBS + cells) controls were included for all experiments.

2.4. Fumonisin B_1 release assay

Pelleted cells with bound FB_1 (as described above) were suspended in 1 ml of 0.022 M PBS pH 7.4 or acetonitrile-water (1:1 v/v) and incubated for 30 min at 37 °C with shaking. After that, the microorganisms were pelleted by centrifugation, and the supernatant containing the released FB_1 was collected and stored at −20 °C for later HPLC analysis. This process was repeated five times.

2.5. Aflatoxin B_1 binding assay

The AFB_1 binding assay was performed according to Bueno et al. (2007). Briefly, stock solution of solid AFB_1 (Sigma, St. Louis, MO,

USA) was suspended in benzene–acetonitrile (97:3 v/v) to obtain an AFB_1 concentration of 1 mg/ml. The benzene–acetonitrile was evaporated by heating at 70 °C for 10 min, and 50 μl of methanol was added and brought to final volume with PBS, pH 7.4. Cells were washed twice with PBS and incubated at 37 °C for 30 min in a shaking bath with 1 ml of PBS containing AFB_1 (1.5; 3.75; 7.5; 15 μg/ml). Then, cells were pelleted by centrifugation at 5000 *g* for 15 min at room temperature, and the supernatant containing unbound AFB_1 was collected and stored at −20 °C until HPLC analysis. Positive (PBS + mycotoxin) and negative (PBS + cells) controls were included for all experiments.

2.6. Binding assay for fumonisin B_1 and aflatoxin B_1 in co-occurrence

Binding assays for AFB1 and FB1 in co-occurrence were performed as described above for each toxin. In this case, the mycotoxins were added simultaneously into the PBS solution. The quantification of AFB1 and FB1, was carried out by HPLC analysis according to Trucksess et al. (1994) and Shephard et al. (1990) modified by Doko et al. (1995), respectively.

2.7. Efficiency parameters

To further study the interaction between the two mycotoxins, we applied the model developed by Bueno et al. (2007), which was proposed to explain the process of AFB1 adsorption by LAB and yeast strains. Briefly, this model considers the attachment of AFB1 molecules to the microorganism surface. The relationship between the amounts of the AFB1 at the microorganism surface as a function of its solution concentration is described by an adsorption isotherm, which is linear at the beginning then transitioning to a plateau. This type of isotherm can be described by the following equation:

$$\text{Adsorption} = M[\text{toxin}] \times K_{eq}/1 + [\text{toxin}]K_{eq}.$$

where M is the maximum number of adsorption sites per microorganism, and K_{eq} (expressed in liters per mole) is equivalent to the affinity of toxin molecules for the adsorption sites. The linearized form of the isotherm is the double-reciprocal plot from the saturation curve, and from the slope and intercept of the resulting line, factors M and K_{eq} can be determined. The most efficient microorganism would be that having maximal M and K_{eq} values or simply the higher product of them ($M \times K_{eq}$). We analyzed the values of these parameters (M and K_{eq}) obtained for the yeast and the LAB, in the absence and presence of two different concentrations of FB_1 (20 and 100 μg/ml), in order to evaluate whether the presence of FB_1 changes the values of M and/or K_{eq} involved in the removal of AFB_1.

2.8. Quantification of fumonisin B_1 by HPLC

FB_1 analysis was performed according to Shephard et al. (1990) and modified by Doko et al. (1995). Fumonisin B_1 was quantified by reversed-phase HPLC with Hewlett Packard series 1100 HPLC equipment, with fluorescence detection (Hewlett Packard 1046 A). The wavelengths used for excitation and emission were 335 nm and 440 nm, respectively. An analytical reversed phase column C18 (150 mm × 4.6 mm internal diameter and 5 μm particle size) connected to a precolumn C18 (20 mm × 4.6 mm and 5 μm particle size) was also used. The mobile phase was a mixture of methanol:0.1 M sodium phosphate di-hydrogenated (75:25 v/v) with the pH being set at 3.35 ± 0.2 with orthophosphoric acid, at a flow rate of 1.5 ml/min. The retention time was around 6 min. Standard curves were prepared with different levels of FB_1 (Sigma, St. Louis, MO, USA) in PBS (pH 7.4). The toxin was quantified by correlating peak areas of samples with those of the standard curves. The detection limit of the technique was 1 ng/g.

Samples of the supernatants and standard of FB_1 were derivatized prior to injection; an aliquot of 50 μl was derivatized with 200 μl of o-phthaldialdehyde (OPA) solution obtained by adding 5 ml 0.1 M sodium tetraborate and 50 μl 2-mercaptoethanol to 1 ml of methanol containing 40 mg of OPA. A recovery experiment was carried out on a series of PBS solutions spiked separately with FB_1 standards (3.125; 25; 50 μg/ml). The experiments were done in duplicate. The mean recovery was 90%.

2.9. Quantification of aflatoxin B_1 by HPLC

AFB_1 analysis was performed by HPLC according to the methodology proposed by Trucksess et al. (1994). AFB_1 was quantified by reversed-phase HPLC with Hewlett Packard series 1100 HPLC equipment, with fluorescence detection (Hewlett Packard 1046 A): λ exc 360 nm; λ em 440 nm and a chromatographic column: C18 column (Supelcosil LC-ABZ, Supelco; 150 × 4.6 mm, 5 μm particle size), connected to a precolumn (Supelguard LC-ABZ, Supelco; 20 × 4.6 mm, 5 μm particle size). Methanol–acetonitrile–water (1:1:4 v/v) was used as the mobile phase at a flow rate of 1.5 ml/min. The retention time was around 5 min. Standard curves were prepared with different levels of AFB_1 (Sigma, St. Louis, MO, USA) in PBS (pH 7.4). The toxin was quantified by correlating peak areas of samples with those of the standard curves. The detection limit of the technique was 1 ng/g. Samples of the supernatants and standard of AFB_1 were derivatized prior to injection, an aliquot of 200 μl of the sample was derivatized with 700 μl trifluoroacetic acid-acetic acid-water (20:10:70 v/v). A recovery experiment was carried out on a series of PBS spiked separately with AFB_1 standards (3.75; 7.5; 15 μg/ml). The experiments were done in duplicate. The mean recovery was 85%.

2.10. Statistical analysis

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

The experiments were done following the flow diagram described in Fig. 1.

3. Results

3.1. Removal of fumonisin B_1 from liquid medium

Fig. 2 shows FB_1 binding by *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 from liquid medium, assayed at different toxin concentrations. Both microorganisms were able to bind the mycotoxin and the level of binding appeared to vary between the species indicating the microorganism specific nature of binding. The percentage of FB_1 removed did not decrease when the toxin concentration increased, as it was similar at all concentrations tested: ~60% and ~20% for yeast and LAB strains respectively.

3.2. Study of microorganism–fumonisin B_1 interaction

In order to establish the mechanism of the removal of FB_1 by *L. acidophilus* 24 and *S. cerevisiae* CECT 1891 we conducted several experiments in which were studied: *i*) the microorganism–FB_1 time contact, *ii*) cell viability, *iii*) concentration of microorganisms, *iv*) release of FB_1 bound to cells and *v*) importance of the microorganism cell wall.

To ascertain if the microorganism–FB_1 interaction was influenced by the incubation time, the removal of FB_1 was evaluated at different periods of time and the results obtained are shown in Table 1. The

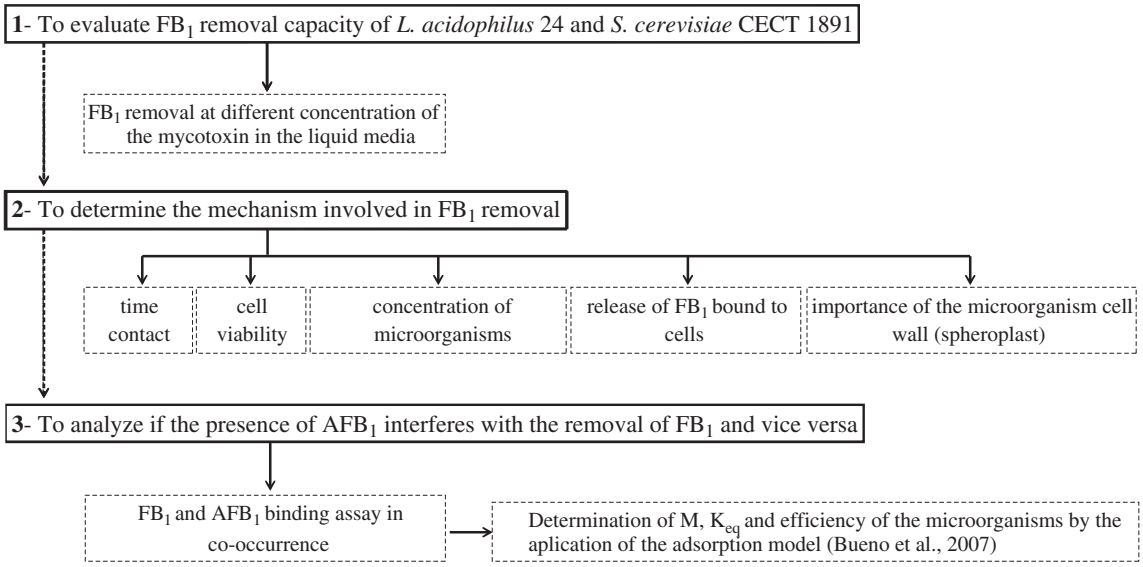

Fig. 1. Flow diagram outlining the experimental design.

incubation time did not affect the elimination of the toxin as no significant differences in the amount of FB_1 removed by *L. acidophilus* 24 and *S. cerevisiae* CECT 1891 were observed. Furthermore, the process was fast, since in 1 min the microorganisms removed the same amount of mycotoxin as in 4.5 h. When the FB_1 removal was analyzed as a function of cell viability, the results showed that nonviable cells (heat treatment) did not decrease their FB_1 binding capacity. On the contrary, the capacities of LAB and yeast heat treated cells were increased 2 and 1.5 times, respectively (Table 1).

In order to prove the effect of cell numbers on the removal of FB_1, we carried out an experiment using an increased microorganism concentration with a fixed amount of mycotoxin (Fig. 3). The results demonstrated that FB_1 binding was dependent on cellular concentration and that both microorganisms showed saturation. The cell increase was never sufficient to bind all the toxins, indicating that the process reached equilibrium between bound toxins (occupied sites) and unbound toxins (free sites). *S. cerevisiae* CECT 1891 was more efficient in FB_1 removal than the LAB strain, since minimum concentrations of ~1×10^7 cell/ml and ~8×10^9 cell/ml were required for 50% removal by *S. cerevisiae* CECT 1891 and *L. acidophilus* 24, respectively.

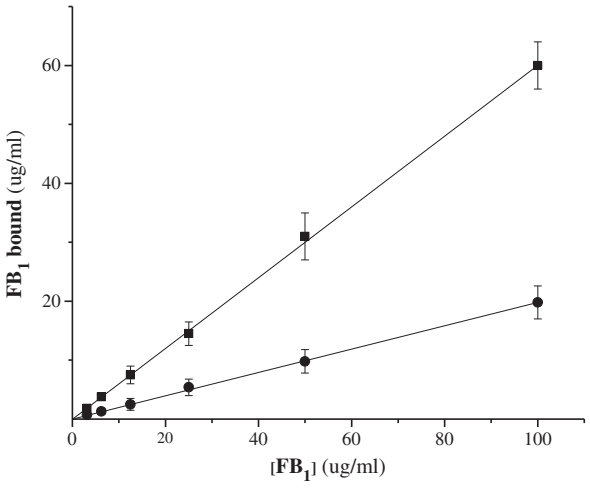

Fig. 2. Removal of FB_1 by (■) *Saccharomyces cerevisiae* CECT 1891 (3.6×10^7 cells/ml) and (●) *Lactobacillus acidophilus* 24 (6.5×10^8 cells/ml). Aliquots of 1 ml of total cells were suspended in PBS in the presence of 3.125; 6.25; 12.5; 25; 50 and 100 μg/ml of FB_1. Results are means ± standard deviations for triplicate samples.

The stability of the FB_1–microorganism complex was studied by the application of five successive washings (each 30 min) with PBS or acetonitrile:water, of the cellular pellets that previously had bound the mycotoxin. Washings using acetonitrile:water released about 50% for *L. acidophilus* 24 and 20–25% for *S. cerevisiae* CECT 1891 of the FB_1 previously bound (Table 2). When removal of the toxin was attempted with PBS, the FB_1 released from cells was lower for both microorganisms, approximately 10% for the LAB and from undetectable to approximately 5% for the yeast, from the total FB_1 bound.

In order to evaluate whether components of the microorganisms cell wall would be involved in the removal of FB_1, we determined the FB_1 binding with three different samples: *i*) whole cells of *S. cerevisiae* CECT 1891 (cell control), *ii*) spheroplasts of *S. cerevisiae* CECT1891 and *iii*) a concentrate of the supernatant from spheroplasts corresponding to 10^7 cells.

As shown in Table 3, neither spheroplasts nor their supernatants were able to remove FB_1 from liquid medium, since very low uptake rates did not change when the concentration of FB_1 in the medium was increased 5 times, suggesting that this binding was nonspecific. Similar results were obtained with spheroplasts from *L. acidophilus* 24 (data not shown).

3.3. Removal of fumonisin B_1 and aflatoxin B_1 when both mycotoxins co-occur in the medium

The possibility of a competition effect was tested by comparing the adsorption of the two mycotoxins separately or together. The

Table 1
Effects of incubation time and cell viability on FB_1 binding.

Time (min)	Fumonisin B_1 binding (μg/ml)			
	L. acidophilus 24[1]		*S. cerevisiae* CECT 1891[2]	
	Viable[3]	Nonviable[3]	Viable[3]	Nonviable[3]
1	3.6 ± 0.3^a	7.7 ± 0.4^b	8.7 ± 0.4^c	12.2 ± 0.2^d
30	3.2 ± 0.2^a	7.6 ± 0.5^b	8.8 ± 0.5^c	12.1 ± 0.3^d
60	3.8 ± 0.3^a	8.1 ± 0.6^b	8.1 ± 0.3^c	11.9 ± 0.5^d
270	3.3 ± 0.2^a	7.5 ± 0.3^b	8.2 ± 0.3^c	12.3 ± 0.4^d

L. acidophilus 24 (4.9×10^8 cells/ml) and *S. cerevisiae* CECT 1891 (3.6×10^7 cells/ml) were incubated at 37 °C at the indicated time (column 1) with FB_1 at a concentration of 15 μg/ml. Results are means ± standard deviations for triplicate samples. [1,2]Values within a row with the same letter are not significantly different ($P<0.05$). [3]There are no significant differences ($P<0.05$) in the mean values of each column.

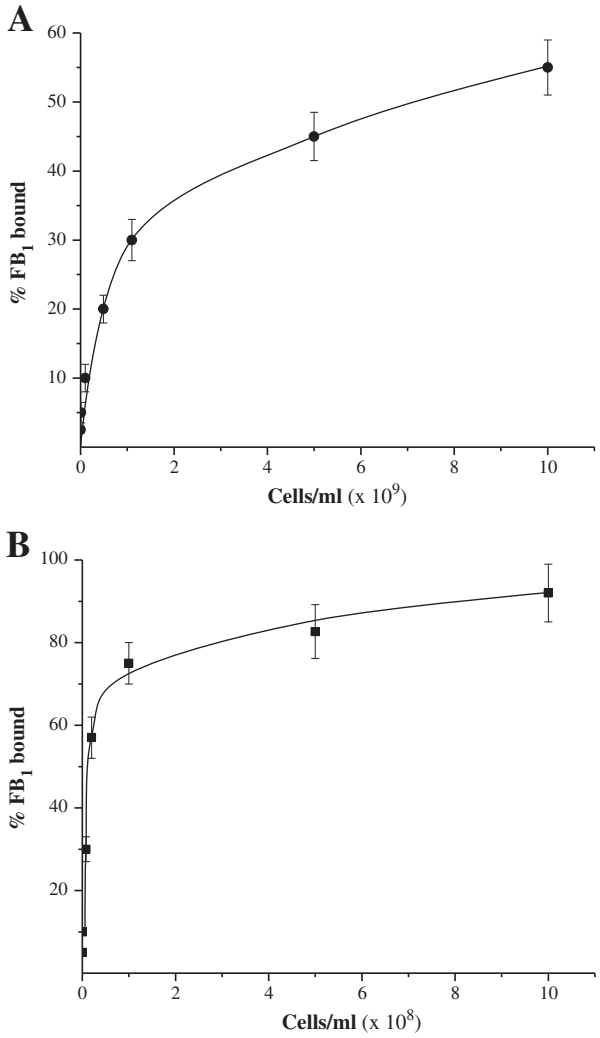

Fig. 3. Effect of microorganism concentration on FB_1 removal by (A) *Lactobacillus acidophilus* 24 and (B) *Saccharomyces cerevisiae* CECT 1891.The percentage of FB_1 bound to cells was calculated as the difference between the total FB_1 (20 μg/ml) and the amount of free FB_1 (supernatant). Results are means ± standard deviations for triplicate samples.

data from Table 4 show that in absence or presence of AFB_1 (up to 15 μg/ml), the capacity to remove FB_1, at two different concentrations (20 and 100 μg/ml), was not modified either for the yeast strain or for the LAB strain. Conversely, when we evaluated if the presence of FB_1 in the medium interfered with the AFB_1 binding ability, no effect was observed (Table 5).

The results shown in Table 6 indicated that *S. cerevisiae* CECT 1891 in the presence of FB_1, did not change its value of M, but the values of K_{eq} were doubled, although they remained within the same order. Similar results were obtained when the studies were done with *L. acidophilus* 24 (data not shown).

Table 3
FB_1 binding by cells, spheroplasts and supernatant of spheroplasts obtained from *S. cerevisiae* CECT 1891.

Sample[1]	FB_1 binding[3]			
	20 μg/ml[2]		100 μg/ml[2]	
	(μg ml^{-1})	(%)	(μg ml^{-1})	(%)
Cell control	12.14 ± 1.47[b]	62.3	58.68 ± 2.97[a]	58.7
Spheroplasts	0.71 ± 0.38[c]	3.6	1.14 ± 0.44[c]	1.1
Supernatant of spheroplasts	0.71 ± 0.36[c]	3.6	0.98 ± 0.41[c]	1.0

[1] Cells and spheroplasts: 10^7 ml^{-1}.
[2] FB_1 concentration in liquid media. Values are means ± standard deviations from triplicate experiments.
[3] Values corresponding to the same letter are not significantly different ($P<0.05$).

4. Discussion

Mycotoxin sequestration in the gastrointestinal tract by adsorbing agents such as microorganisms could be a promising strategy to protect against the toxic effect of these food contaminants (Firmin et al., 2010).

Although LAB and yeast have been studied as mycotoxin decontaminating agents (Bueno et al., 2007; Haskard et al., 2000; Pizzolitto et al., 2011; Shetty et al., 2007), the nature of FB_1 binding is still poorly understood and the microorganism–FB_1 interaction needs to be better characterized to optimize the selection of strains as mycotoxin removal agent. In this work, *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 previously reported to be efficient AFB_1 adsorbent agents (Bueno et al., 2007; Pizzolitto et al., 2011), were studied for their ability to bind FB_1. In addition, we identified the nature of the mechanism involved (physical or chemical) in FB_1 binding process and we characterized the removal in terms of binding conditions of the selected strains.

The results showed that *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 were able to bind high amounts of FB_1 even at the highest concentration tested (100 μg/ml). Similar results were reported by Shetty et al. (2007) for AFB_1 removal by *S. cerevisiae* strains. The binding was not saturated showing the high efficiency of these strains. *S. cerevisiae* CECT 1891 strain was always more effective than the LAB strain, demonstrating microorganism specificity in the removal process.

The present study showed that the process involved in the fumonisin–microorganism interaction needs very little time (1 min), suggesting that neither entrance of FB_1 into cell nor its metabolic conversion occurs. This hypothesis was confirmed when the effect of cell viability in FB_1 removal was evaluated. Heat treatment enhanced the binding and autoclaving resulted in highest binding abilities. The effectiveness of nonviable cells in binding

Table 2
FB_1 bound and released by *L. acidophilus* 24 and *S. cerevisiae* CECT 1891.

[FB_1] (μg/ml)	Fumonisin B_1 binding (μg/ml)		Fumonisin B_1 released[a] (%)			
			PBS		ACN:H_2O	
	L. acidophilus 24	*S. cerevisiae* CECT 1891	*L. acidophilus* 24	*S. cerevisiae* CECT 1891	*L. acidophilus* 24	*S. cerevisiae* CECT 1891
12.5	3.0 ± 1.0	8.0 ± 2.1	12.0 ± 3.8	ND	56.0 ± 6.0	ND
25	5.9 ± 1.2	13.1 ± 1.8	13.0 ± 3.2	ND	54.0 ± 5.1	20.0 ± 4.5
50	10.3 ± 1.7	29.2 ± 2.4	15.0 ± 3.8	5.5 ± 2.1	48.0 ± 3.9	25.0 ± 4.1
100	21.2 ± 3.1	61.0 ± 2.9	10.0 ± 3.1	6.2 ± 3.2	47.0 ± 4.8	23.0 ± 3.8

Cell number: 6.5×10^8 cells/ml for *L. acidophilus* 24 and 3.6×10^7 cells/ml for *S. cerevisiae* CECT 1891.
[a] The FB_1 released were expressed as a percentage of the total FB_1 bound. Values are means ± standard deviations from triplicate experiments.

Table 4
FB_1 removal in presence of AFB_1.

[AFB_1] (μg/ml)	FB_1 binding[a] (μg/ml)			
	L. acidophilus 24		*S. cerevisiae* CECT 1891	
	20[b]	100[b]	20[b]	100[b]
0	3.5 ± 0.9	18.1 ± 2.6	10.3 ± 1.5	50.5 ± 3.8
1.5	3.6 ± 1.1	17.3 ± 2.8	10.0 ± 1.3	48.9 ± 2.9
3.75	3.4 ± 0.9	18.0 ± 2.2	9.8 ± 1.7	50.0 ± 4.1
7.5	3.3 ± 0.8	17.2 ± 2.3	9.9 ± 1.8	47.5 ± 3.7
15	3.4 ± 1.1	17.4 ± 1.9	11.0 ± 1.4	49.5 ± 3.9

[a] There are no significant differences in the mean values of each column ($P<0.05$).
[b] FB_1 concentration in liquid media (μg/ml). The samples (2.0×10^8 cells/ml for *L. acidophilus* 24 and 2.4×10^7 cells/ml for *S. cerevisiae* CECT 1891) were suspended in 1 ml PBS containing 20 or 100 μg/ml of FB_1 in presence of the AFB_1 concentrations indicated in column 1. Values are means ± standard deviations from triplicate experiments.

indicates that FB_1 is not removed by metabolism. This physical treatment may change the original binding sites of the viable microorganisms, modifying them favorably and exposing new binding sites. This result is in agreement with previous studies reported by Niderkorn et al. (2006) that indicated that biodegradation did not appear to be the mode of action, as no toxin derivatives were observed and removal was not impaired in nonviable bacteria.

Since metabolic activation cannot occur, covalent interactions cannot be responsible for the binding either. Non-covalent interactions are proposed to occur with microbial surface components (Haskard et al., 2000).

In order to determine the efficiency of the microorganisms in fumonisin removal, total microorganism number (cells/ml) rather than viable cells (CFU/ml) should be used to estimate cell concentration. The elimination of FB_1 from liquid medium was, in contrast with the results reported by Niderkorn et al. (2006), strongly dependent on microorganism concentration in the medium; this relationship was linear at low cell concentrations then transitioned to a plateau, indicating reversibility of the process involved, since the cell number increase was not ever sufficient to remove all molecules of toxin present in the sample. Reversibility of the process was confirmed by release of FB_1 back into the solution from the microorganism–FB_1 complexes by washing the microorganisms, confirming that binding involved was weak non-covalent interaction. It is interesting to note that while the yeast was always more efficient in FB_1 binding than *L. acidophilus* 24, it also released lower amounts of mycotoxin than the LAB, indicating that the complex formed with the yeast strain was the most stable. The stability of the microorganism–mycotoxin complex in the gastro-intestinal tract is essential for the practical application of this technology (Niderkorn et al., 2006). However, probiotics with AFB_1 bound to their surfaces are less likely to adhere to the intestinal wall and prolong exposure to dietary AFB_1. Hence, specific probiotics may be a potent and safe means to reduce absorption

Table 5
AFB_1 removal in presence of FB_1.

[FB_1] (μg/ml)	AFB_1 binding[a] (μg/ml)			
	L. acidophilus 24		*S. cerevisiae* CECT 1891	
	3.75[b]	15[b]	3.75[b]	15[b]
0	0.8 ± 0.1	2.4 ± 0.5	2.8 ± 0.4	10.1 ± 1.4
20	0.9 ± 0.2	2.5 ± 0.6	2.9 ± 0.6	9.2 ± 0.9
100	0.7 ± 0.2	2.3 ± 0.4	2.9 ± 0.8	9.1 ± 0.8

[a] There are no significant differences in the mean values of each column ($P<0.05$).
[b] AFB_1 concentration in liquid media (μg/ml). The samples (2.0×10^8 cells/ml for *L. acidophilus* 24 and 2.4×10^7 cells/ml for *S. cerevisiae* CECT 1891) were suspended in 1 ml PBS containing 3.75 or 15 μg/ml of AFB_1 in presence of the FB_1 concentrations indicated in column 1. Values are means ± standard deviations from triplicate experiments.

Table 6
Efficiency parameters (M and K_{eq}) for the adsorption of AFB_1 by *S. cerevisiae* CECT 1891.

Conditions[a]	M (sites/cells) × 10^9	K_{eq} (M^{-1}) × 10^5	Efficiency (M × K_{eq}) × 10^{14}
AFB_1	1.03	1.1	1.13
$AFB_1 + FB_1$ (20 μg/ml)	1.04	2.3	2.39
$AFB_1 + FB_1$ (100 μg/ml)	0.96	2.3	2.21

[a] Cells were suspended in 1 ml PBS with increasing concentrations of AFB_1 (1.5; 3.75; 7.5 and 15 mg/ml) in the absence or presence of FB_1 (20 and 100 mg/ml). Total binding sites per cell (M), equilibrium constant (K_{eq}), and adsorption efficiency (M × K_{eq}) were calculated according to Bueno et al. (2007).

and increase excretion of dietary AFB_1 from the body (Gratz et al., 2004).

The nature of cell wall components involved in mycotoxin binding is still not clear. The assays with spheroplasts indicated that the cell wall components involved in FB_1–microorganism interaction must maintain their structure in order to effectively remove the toxin. These results confirm the role of a cell wall-related physical phenomenon as opposed to a metabolic degradation reaction, and are consistent with the results reported by Niderkorn et al. (2009), who attributed FB_1 binding by LAB to peptidoglycans.

The mechanism involved in FB_1 removal by *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 strains has been demonstrated to be a physical phenomenon and FB_1 is bound to the microorganisms by weak non-covalent interactions, such as associating with hydrophobic pockets on the microorganism surface. Our results are consistent with those reported by Haskard et al. (2000), Bueno et al. (2007) for AFB_1 and with Niderkorn et al. (2006) for FB_1, that indicated binding at the bacterial cell wall as the mechanism of removal of mycotoxins by LAB.

To identify the type of chemical moieties and interactions involved in AFB_1 and FB_1 binding *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 were subjected to chemical and enzymatic treatments and the possible effects on mycotoxins studied (unpublished results). Preliminary results are consistent with those reported by other authors. Haskard et al. (2000) described that similar binding of AFB_1 to viable, heat-killed and acid-killed *Lactobacillus rhamnosus* GG occurred. The effects of pronase E, lipase and *m*-periodate on AFB_1 binding and release were consistent with AFB_1 binding predominantly to carbohydrate components of the bacteria. The effect of urea suggested that hydrophobic interactions play a major role in binding. Increasing concentration (0.01–1 M) of NaCl or $CaCl_2$ had minor effects on AFB_1 binding suggesting some involvement of electrostatic interactions. The increase in binding observed with heat- and acid-treated bacteria was also reported for El-Nezami et al. (1998) for AFB_1 and it is known that these treatments degrade the surface of the cell wall. Lahtinen et al. (2004) suggested that cell wall peptidoglycans are responsible for AFB_1 removal by LAB, whereas Raju and Devegowda (2000) attributed the aflatoxin binding by yeast cell walls to mannan oligosaccharides. On the other hand, Niderkorn et al. (2009) reported that treatments affecting the bacterial wall polysaccharides, lipids and proteins increased binding, while those degrading peptidoglycan partially decreased it. In addition, purified peptidoglycan from Gram positive bacteria bound FB_1 in a manner analogous to that of intact LAB. They also reported that at least one tricarballylic acid chain of FB_1 played a significant role in binding as hydrolyzed FB had less affinity for LAB.

According to an integrated synthesis of the results reported above, it is clear that: (i) *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 are able to remove FB_1 from liquid medium; (ii) this removal is a fast and reversible process; (iii) cell viability is not necessary for FB_1 binding; (iv) the amount of FB_1 removed is both toxin- and microorganism concentration- dependent; (v) the process does not involve chemical modification of FB_1 molecules; and (vi) the cell wall structural integrity of the microorganisms is required for the FB_1 removal. Briefly, the process involved is, by nature, reversible and the kinetics are rapid. Consequently, we propose that the mechanism involved in the

removal of FB_1 by *S. cerevisiae* CECT 1891 and *L. acidophilus* 24 is a physical adsorption (physisorption) of the toxin molecule to cell wall components of the microorganisms.

Although our FB_1 binding experiments did not show saturation, even with high fumonisin concentrations (100 μg/ml), we propose that the same model developed by Bueno et al. (2007) for the AFB_1 binding, could be applied for the removal of FB_1, because with higher FB_1 concentrations in the medium, saturation would be observed and the parameters M and K_{eq} and the efficiency ($M \times K_{eq}$) to remove FB_1 from the medium, of the tested microorganism could be calculated. Comparing the results reported for the interaction AFB_1–*L. acidophilus* 24 and AFB_1–*S. cerevisiae* CECT 1891 (Bueno et al., 2007; Pizzolitto et al., 2011), with those obtained in this work carried out with FB_1, could indicate that the microorganisms had different values of M and K_{eq} when interacting with either AFB_1 or FB_1. In fact, results showed that AFB_1 released rates were twice as high as those obtained with FB_1, indicating that the K_{eq} of the process with FB_1 was higher than the K_{eq} obtained with AFB_1. On the other hand, aflatoxin and fumonisin binding assays according to their concentrations in the medium, indicated that for similar number of cells, saturation with a concentration of AFB_1 molecules (0–10 μg/ml and MW = 312.27) was four times lower than FB_1 (0–100 μg/ml and MW = 721.83), whereas with FB_1 concentrations saturation was not observed. These results give an indication that the M value for FB_1 is higher than the obtained for AFB_1. Briefly, saturation was not reached with the FB_1 concentrations employed, and when compared with the AFB_1 results obtained with the same microorganisms, the values of M and K_{eq} for FB_1 were greater than those obtained with AFB_1. For this reason the microorganisms showed higher efficiency in the removal of FB_1.

The present study reports, the removal of FB_1 and AFB_1 in co-occurrence from liquid medium by the selected strains. As we have proposed, FB_1 and AFB_1 are removed by adsorption to cell wall components of the microorganisms and through the same mechanism, therefore testing co-occurrence of both mycotoxins were needed to determine whether FB_1 interferes with the removal of AFB_1, and vice versa. The results showed that in presence of FB_1, the number of AFB_1 binding sites per cell (M) did not change, and K_{eq} slightly increased, thus FB_1 and AFB_1 are bound to cell wall components of the microorganisms, but at different sites, and therefore there was no competition for the binding sites. The increased value of the K_{eq}, although slight, indicates that in the presence of FB_1, the microorganism–AFB_1 complex will be more stable and when it is exposed to consecutive washings, it will release less toxin, resulting in a positive effect. On the other hand, it is interesting to note that the presence of AFB_1 did not modify the values obtained in the removal of FB_1, thus given some indication that when both mycotoxins co-occur in the medium, their removal would be as if they were alone.

Co-exposure to multiple mycotoxins is a cause of concern because so many have been shown to be potent toxic agents with diverse effects which may be synergistic. It is logical to raise this issue because any single compound may affect dissimilar reactions within a biological system, while displaying antagonistic, additive, or synergistic interactions with other compounds (Carpenter et al., 1998). The co-contamination of foodstuffs with AFB_1 and FB_1 is well known and has been implicated in the development of human hepatocellular carcinoma in high risk areas around the world (Li et al., 2001; Ueno et al., 1997; Wang et al., 1995). Gelderblom et al. (2002) established that when rats were treated in a sequential manner with AFB_1 and FB_1, there was a significantly increased cancer initiating potency. In addition to the cancer promoting activity of FB_1 on AFB_1 initiated hepatocytes, AFB_1 pre-treatment seemed to enhance the FB_1 initiating potency, presumably by rendering the liver more susceptible to the toxic effects of FB_1. Studies performed by Theumer et al. (2008) indicated that the co-exposure to fumonisins and AFB_1 produced a higher liver toxicity, compared with individual administration, inducing apoptosis and mitotic hepatocytes. There was an inversion of the typical sphinganine to sphingosine (Sa/So) ratio in rats fed on the culture material as well as in those subjected to a diet co-contamined with fumonisins and AFB_1. Results reported by Mckean et al. (2006) demonstrate that these two toxins interacted to produce alterations in the toxic responses with a strong additive interaction noted in the cases of F344 rats and mosquito fish.

In conclusion, in the present work the main mechanism involved in FB_1 removal for *L. acidophilus* 24 and *S. cerevisiae* CECT 1891 strains was elucidated and is due to physical adsorption of the mycotoxin to a component of the microorganism cell wall. More work is required to completely unravel the mechanism of binding, however some significant factors affecting the binding efficiency have been identified in the present study. The selected strains were able to remove FB_1 and AFB_1 from liquid medium independently, even with co-occurrence of both mycotoxins and they did not compete for binding sites on the microorganism cell wall. Furthermore, the presence of one toxin did not modify the efficiency of the organism in the removal of another mycotoxin. The characteristics of the binding mechanism identified in this report may be useful for optimization of mycotoxin binding and provide an important contribution to research into microorganisms with ability to remove these secondary metabolites.

Acknowledgments

The authors thank Dr. L. Barberis and Dr. L. Pascual for providing us with the *L. acidophilus* 24 isolate. This work was carried out thanks to grants from SECYT-UNRC, PICT, Ministerio de Ciencia y Tecnología de Córdoba and PICT-CNPq. Romina Pizzolitto thanks CONICET-MinCyT Cba. for fellowship support.

References

Abbas, H.K., Gelderblom, W.C., Cawood, M.E., Shier, W.T., 1993. Biological activities of fumonisins, mycotoxins from *Fusarium moniliforme*, in jimsonweed (*Datura stramonium* L.) and mammalian cell cultures. Toxicon 31, 345–353.

Bueno, D., Casale, C., Pizzolitto, R., Salvano, M., Oliver, G., 2007. Physical adsorption of aflatoxin B_1 by lactic acid bacteria and *Saccharomyces cerevisiae*: a theoretical model. Journal of Food Protection 70, 2148–2154.

Carpenter, D.O., Arcaro, K.F., Bush, B., Niemi, W.D., Pang, S., Vakharia, D.D., 1998. Human health and chemical mixtures: an overview. Environmental Health Perspectives 106, 1263–1270.

CAST (Council for Agricultural Science, Technology), 2003. Mycotoxins: Risks in Plant, Animal and Human Systems, Task Force 478 Report No. 139 Ames, Iowa, USA.

Doko, B., Rapior, S., Visconti, A., Schjoth, J., 1995. Incidence and levels of fumonisin contamination in maize genotypes grown in Europe and Africa. Journal of Agricultural and Food Chemistry 43, 429–434.

El-Nezami, H., Kankaanpää, P., Salminen, S., Ahokas, J., 1998. Physicochemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin fron contaminated media. Journal of Food Protection 61, 466–468.

El-Nezami, H., Polychronaki, N., Salminen, S., Mykkanen, H., 2002a. Binding rather than metabolism may explain the interaction of two food-grade *Lactobacillus* strains with zearalenone and its derivative α-zearalenol. Applied and Environmental Microbiology 68, 3545–3549.

El-Nezami, H.S., Chrevatidis, A., Auriola, S., Salminen, S., Mykkanen, H., 2002b. Removal of common *Fusarium* toxins in vitro by strains of *Lactobacillus* and *Propionibacterium*. Food Additives and Contaminants 19, 680–686.

Firmin, S., Gandia, P., Morgavi, D., Houin, G., Jouany, J., Bertin, G., Boudra, H., 2010. Modification of aflatoxin B_1 and ochratoxin A toxicokinetics in rats administered a yeast cell wall preparation. Food Additives and Contaminants 27, 1153–1160.

Firmin, S., Morgavi, D.P., Yiannikouris, A., Boudra, H., 2011. Effectiveness of modified yeast cell wall extracts to reduce aflatoxin B_1 absorption in dairy ewes. Journal of Dairy Science 94, 5611–5619.

Gelderblom, W.C.A., Marasas, W.F.O., Lebepe-Mazur, S., 2002. Interaction of fumonisin B_1 and aflatoxin B_1 in a short term carcinogenesis model in rat liver. Toxicology 171, 161–173.

Gratz, S., Mykkänen, H., Ouwehand, A.C., Juvonen, R., Salminen, S., El-Nezami, H., 2004. Intestinal mucus alters the ability of probiotic bacteria to bind aflatoxin B_1 in vitro. Applied and Environmental Microbiology 70, 6306–6308.

Gratz, S., Wu, Q.K., El-Nezami, H., Juvonen, R.O., Mykkänen, H., Turner, P.C., 2007. *Lactobacillus rhamnosus* strain GG reduces aflatoxin B_1 transport, metabolism and toxicity in caco-2 cells. Applied and Environmental Microbiology 73, 3958–3964.

Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E., Cole Jr., J.R., 1990. Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*. Journal of Veterinary Diagnostic Investigation 2, 217–221.

Haskard, C., Binnion, C., Ahokas, J., 2000. Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. Chemico-Biological Interactions 128, 39–49.

Hernandez-Mendoza, A., Guzmán de Peña, D., Garcia, H.S., 2009. Key role of teichoic acids on aflatoxin B_1 binding by probiotic bacteria. Journal of Applied Microbiology 107, 395–403.

International Agency for Research on Cancer (IARC), 2002. IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans: Some Traditional Herbal Medicines, some Mycotoxins, Naphthalene and Styrene, 82. International Agency for Research on Cancer, Lyon, France, pp. 301–366.

Keller, S.E., Sullivan, T.M., Chirtel, S., 1997. Factors affecting the growth of *Fusarium proliferatum* and the production of fumonisin B_1: oxygen and pH. Journal of Industrial Microbiology and Biotechnology 19, 305–309.

Krska, R., Welzig, E., Boudra, H., 2007. Analysis of *Fusarium* toxins in feed. Animal Feed Science and Technology 137, 241–264.

Lahtinen, S.J., Haskard, C.A., Ouwehand, A.C., Salminen, S.J., Ahokas, J.T., 2004. Binding of aflatoxin B_1 to cell wall components of *Lactobacillus rhamnosus* strain GG. Food Additives and Contaminants 21, 158–164.

Lamprecht, S.C., Marasas, W.F.O., Alberts, J.F., Cawood, M.E., Gelderblom, W.C.A., Shephard, G.S., Thiel, P.G., Calitz, F.J., 1994. Phytotoxicity of fumonisins and TA-toxin to corn and tomato. Phytopathology 84, 383–391.

Li, F.Q., Yoshizawa, T., Kawamura, O., Luo, X.Y., Li, Y.W., 2001. Aflatoxins and fumonisins in corn from the high-incidence area for human hepatocellular carcinoma in Guangxi, China. Journal of Agricultural and Food Chemistry 49, 4122–4126.

McKean, C., Tang, L., Tang, M., Billam, M., Wang, Z., Theodorakis, C., Kendall, R., Wang, J., 2006. Comparative acute and combinative toxicity of aflatoxin B_1 and fumonisin B_1 in animals and human cells. Food and Chemical Toxicology 44, 868–876.

Niderkorn, V., Boudra, H., Morgavi, D.P., 2006. Binding of *Fusarium* mycotoxins by fermentative bacteria *in vitro*. Journal of Applied Microbiology 101, 849–856.

Niderkorn, V., Morgavi, D.P., Pujos, E., Tissandier, A., Boudra, H., 2007. Screening of fermentative bacteria for their ability to bind and biotransform deoxynivalenol, zearalenone and fumonisins *in vitro* simulated corn silage model. Food Additives and Contaminants 24, 406–415.

Niderkorn, V., Morgavi, D., Aboab, B., Lemaire, M., Boudra, H., 2009. Cell wall component and mycotoxin moieties involved in binding of fumonisin B_1 and B_2 by lactic acid bacteria. Journal of Applied Microbiology 106, 977–985.

Oliveira, G.R., Ribeiro, J.M.M., Fraga, M.E., Cavaglieri, L.R., Direito, G.M., Keller, K.M., Dalcero, A.M., Rosa, C.A.R., 2006. Mycobiota in poultry feeds and natural occurrence of aflatoxins, fumonisins and zearalenone in the Rio de Janeiro State, Brazil. Mycopathologia 162, 355–362.

Ovalle, R., Lim, S.T., Holder, B., Jue, C.K., Moore, C.W., Lipke, P.N., 1998. A spheroplast rate assay for determination of cell wall integrity in yeast. Yeast 14, 1159–1166.

Pietri, A., Zanetti, M., Bertuzzi, T., 2009. Distribution of aflatoxins and fumonisins in dry-milled maize fractions. Food Additives and Contaminants: Part A, Chemistry, Analysis, Control, Exposure and Risk Assessment 26, 372–380.

Pizzolitto, R.P., Bueno, D.J., Armando, M.R., Cavaglieri, L.R., Dalcero, A.M., Salvano, M.A., 2011. Binding of aflatoxin B_1 to lactic acid bacteria and *Saccharomyces cerevisiae in vitro*: A useful model to determine the most efficient microorganism. Aflatoxins—Biochemistry and Molecular Biology. Intech Publications, Croatia, pp. 323–346.

Raju, M., Devegowda, G., 2000. Influence of esterified-glucomannan on performance and organ morphology, serum biochemistry and haematology in broilers exposed to individual and cobined mycotoxicisis. British Poultry Science 41, 640–650.

Riley, R.T., Showker, J.L., Owens, D.L., Ross, P.F., 1997. Disruption of sphingolipid metabolism and induction of equine leukoencephalomalacia by *Fusarium proliferatum* culture material containing fumonisin B_2 or B_3. Environmental Toxicology and Pharmacology 3, 221–228.

Rocha, L.O., Nakai, V.K., Braghini, R., Reis, T.A., Kobashigawa, E., Correa, B., 2009. Mycoflora and co-occurrence of fumonisins and aflatoxins in freshly harvested corn in different regions of Brazil. International Journal of Molecular Sciences 24, 5090–5103.

Shephard, G.S., Sydenham, E.W., Thiel, P.G., Gelderblom, W.C.A., 1990. Quantitative determination of fumonisin B_1 and B_2 by high-performance liquid chromatography with fluorescence detection. Journal of Liquid Chromatography 13, 2077–2087.

Shephard, G.S., Marasas, W.F.O., Leggott, N.L., Yazdanpanah, H., Rahimian, H., Safavi, N., 2000. Natural occurrence of fumonisins in corn from Iran. Journal of Agricultural and Food Chemistry 48, 1860–1864.

Shetty, P., Jespersen, L., 2006. *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends in Food Science and Technology 17, 48–55.

Shetty, P., Hald, B., Jespersen, L., 2007. Surface binding of aflatoxin B_1 by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. International Journal of Food Microbiology 113, 41–46.

Theumer, M., López, A., Aoki, M., Cánepa, M., Rubinstein, H., 2008. Subchronic mycotoxicoses in rats. Histopathological changes and modulation of the sphinganine to sphingosine (Sa/So) ratio imbalance induced by *Fusarium verticillioides* culture material, due to the coexistence of aflatoxin B_1 in the diet. Food and Chemical Toxicology 46, 967–977.

Trucksess, M.W., Stack, M.E., Nesheim, S., Albert, R.H., Romer, T.R., 1994. Multifunctional column coupled with liquid chromatography for determination of aflatoxins B_1, B_2, G_1 and G_2 in corn, almonds, Brazil nuts, peanuts and pistachio nuts: collaborative study. Journal of AOAC International 77, 1512–1521.

Ueno, Y., Iijima, K., Wang, S.D., Sugiura, Y., Sekijima, M., Tanaka, T., Chen, C., Yu, S.Z., 1997. Fumonisins as a possible contributory risk factor for primary liver cancer: a 3-year study of corn harvested in Haimen, China by HPLC and ELISA. Food and Chemistry Toxicology 35, 1143–1150.

Voss, K.A., Smith, G.W., Haschek, W.M., 2007. Fumonisins: toxicokinetics, mechanism of action and toxicity. Animal Feed Science and Technology 137, 299–325.

Wang, D.S., Liang, Y.X., Chau, N.T., Dien, L.D., Tanaka, T., Ueno, Y., 1995. Natural co-occurrence of *Fusarium* toxins and aflatoxin B_1 in corn for feed in North Vietnam. Natural Toxins 3, 445–449.