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ORIGINAL ARTICLE

In vitro aflatoxin B_1 binding capacity by two *Enterococcus faecium* strains isolated from healthy dog faeces

M.G. Fernández Juri, A.M. Dalcero and C.E. Magnoli

Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Río Cuarto, Córdoba, Argentina

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Correspondence
María Guillermina Fernández Juri, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas Fco.-Qcas. y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional 36 Km 601 (5800), Río Cuarto, Córdoba, Argentina.
E-mail: gfernandezj@exa.unrc.edu.ar

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Abstract

Aim: This study evaluated the binding capacity of aflatoxin B_1 (AFB_1) by two *Enterococcus faecium* strains (MF4 and GJ40) isolated from faeces from healthy dogs.

Materials and Methods: The binding assay was performed using 50 and 100 ppb of AFB_1 analysing the effects of the viability, incubation time and pH on AFB_1 binding. Binding stability was determined by washing three times the bacteria-AFB_1 complexes with phosphate buffer saline.

Results: Both GJ40 and MF4 strains have the ability to remove AFB_1 from aqueous solution. Viable cells were slightly more effective in AFB_1 binding than nonviable ones for both strains. *Enterococcus faecium* GJ40 removes 24–27% and 17–24%, and *Ent. faecium* MF4 removes 36–42% and 27–32% of AFB_1 (50 and 100 ppb, respectively) throughout a 48 h incubation period. In general, the removal of AFB_1 was highest at pH 7·00 for both strains. The stability of the bacteria-AFB_1 complex formed was found to be high (up to 50% of AFB_1 remained bounded in bacterial cell after three washes with phosphate buffered saline).

Conclusion: The *Ent. faecium* strains assayed are capable of removing AFB_1 under different conditions *in vitro*.

Significance and Impact of the Study: This is the first AFB_1 binding assay performed with *Ent. faecium* strains isolated from dog faeces, being an interesting strategy for AFB_1 decontamination of pet food.

Introduction

Mycotoxins are food contaminants with harmful impact on human and animal health (CAST, 2003). Domestic dogs play several important roles in modern human society (Suchodolski *et al.* 2004). Pet food elaboration requires the use of appropriate technology to ensure its nutritional value and enhance life quality of pets. Many owners of pet food industries are concerned about the potential risk for mycotoxins contamination in pet foods, as the pets are fed for longer periods of time. Thus, these animals may become more vulnerable to chronic exposure to toxicants, especially to aflatoxins (AFs) which cause acute hepatotoxic and carcinogenic effects in dogs (Binder 2006; Boermans and Leung 2007). Previous works report important percentages of *Aspergillus* section *Flavi* AFs producers strains and samples contaminated with aflatoxin B_1 (AFB_1) in ready-to-eat pet foods (Basalan *et al.* 2004; Leung *et al.* 2006; Campos *et al.* 2008, 2009; Fernández Juri *et al.* 2009a,b). Recently, international legislation establishes AFB_1 limits in pet food of 20–300 ppb in USA and 10–50 ppb in Europe (Aquino and Corrêa 2011). Several procedures are used for mycotoxins decontamination such as physical, chemical and biological methods (Park 1993). Live micro-organisms can decontaminate either by attaching the mycotoxin to their cell wall components or by active internalization and accumulation. Dead cells of microbes can also adsorb mycotoxins. This phenomenon can be exploited in the creation of biofilters for fluid decontamination or probiotics (which have proven binding capacity) to bind and remove the mycotoxin from the intestine (Juodeikiene *et al.* 2012). Yeast and lactic acid bacteria (LAB) cells can bind different molecules on the cell wall surface

(Bolognani *et al.* 1997; Santos *et al.* 2006). The mycotoxins removal is mainly produced by adhesion to cell wall components rather than by covalent binding or metabolism, as the dead cells do not lose their binding ability (Celyk *et al.* 2003; Santin *et al.* 2003; Baptista *et al.* 2004). Several LAB strains of food or animal origin have been tested for their ability to bind AFs and other mycotoxins to their surface (El-Nezami *et al.* 2002; Fuchs *et al.* 2008). Researchers have reported that different bacterial strains are able to bind AFB_1 in significantly different amounts (Peltonen *et al.* 2001). Fuchs *et al.* (2008) showed that LAB strains can remove patulin and ochratoxin A from aqueous solution in different levels. *Enterococcus faecium* is a member of LAB mostly found in nature and has various applications in the processing of some fermented dairy products (Giraffa 2003). Enterococci are used as probiotics in commercial formulation specially destined for animal feeding being mostly produced in foreign countries, not in Argentina. These strains produce antimicrobial substances against different bacteria as well as some fungal strains (Simonetta *et al.* 1997). In a previous work, the inhibitory effect of *Ent. faecium* strains isolated from the normal microbiota of healthy dogs gut on fungal growth parameters, and AFB_1 production by aflatoxigenic strains on *in vitro* assays was determined (Fernández Juri *et al.* 2011). The results obtained in the assay are promising due to the high percentages of AFB_1 production inhibition by *Ent. faecium* strains, even though they were unable to reduce *Aspergillus* strains growth rate. Topcu *et al.* (2010) reported the AFB_1 and patulin detoxification capacity of two commercial probiotic strains (*Ent. faecium* M74 and EF031) from aqueous solution. Both strains assayed had the ability to remove high percentages of the toxins throughout the assays. The viability of the bacteria did not have any significant effect on the detoxification of AFB_1 and patulin. However, there is no information available respect to AFB_1 binding capacity of *Ent. faecium* isolated from healthy dog faeces. Therefore, the aim of this study was to study the AFB_1 binding by two *Ent. faecium* strains isolated from faeces of healthy dogs that inhibit AFB_1 production in high percentages on *in vitro* assays. The effect of cell viability, incubation time and pH in the binding process and the stability of the AFB_1-viable cell were investigated.

Materials and methods

Chemicals and media

Man-Rogosa (MRS) (Britania) was used for *Ent. faecium* strains growth. Aflatoxin B_1 was obtained from Sigma-Aldrich (St. Louis, MO) and phosphate buffer solution (PBS) from Britania (Buenos Aires, Argentina). For the AFB_1 binding assay, solid AFB_1 was suspended in benzene–acetonitrile (97/3, v/v) to obtain an AFB_1 concentration of 1000 ppb, then benzene–acetonitrile was evaporated by nitrogen stream and AFB_1 was resuspended in methanol (stock solution). Two working solutions (50 and 100 ppb) were prepared in PBS at pH 7·00 using the stock solution of AFB_1 in methanol (El-Nezami *et al.* 1998; Haskard *et al.* 2001).

Bacterial strains, culture conditions and count

Cultures of *Ent. faecium* MF4 and GJ40 were isolated from faeces of healthy dogs. Strains selection was based on their capacity of AFB_1 reduction or production inhibition (Fernández Juri *et al.* 2013). *Enterococcus faecium* strains were cultivated in MRS broth (24 h, 37°C) for preparation of overnight cultures. Samples of 1 ml of cultivated bacterial suspensions were decimally diluted in sterile peptone water (0·1%, w/v). Colony forming units (CFU) in overnight cultures were determined by plate counting on MRS Agar. In this study, all incubations were carried out at 37°C, and all centrifugations were at 3000 ***g*** for 15 min (4°C).

Mycotoxin binding assay

Viability assay

Cultures of each strain were divided in two aliquots. Aliquot 1: a volume of the culture broth (1×10^8 bacteria) was transferred into microtubes and centrifuged (viable cells). Aliquot 2: a volume of culture broth (1×10^8 bacteria) was transferred into microtubes then autoclaved at 121°C for 20 min (nonviable cells) and centrifuged. Supernatants were removed, and bacterial pellets (viable and nonviable cells) were washed with deionized water and centrifuged again (Peltonen *et al.* 2001; Topcu and Bulat 2010). For each strain, bacterial pellets (viable and nonviable) were resuspended in 2 ml of AFB_1 working solutions (50 and 100 ppb, pH 7·00), and the AF determination was performed after incubation at 24 h. Mycotoxin solutions without cells were used as controls. All the experiments were carried out by triplicate and repeated three times.

Incubation time effect

For incubation time effect, samples of *Ent. faecium* cells + AFB_1 were mixed in vortex shortly (5 s) and incubated for 48 h on an orbital shaker (New Brunswick Scientific CO., INC., Edison, NJ) with soft agitation. Supernatant samples (500 μl) were collected by centrifugation after incubation for 1, 24 and 48 h, and kept at −20°C for further analysis.

pH effect

For pH effect assay, PBS solution (pH 7·00) containing AFB_1 was adjusted to pH 3·00 and 5·00 with 1 mol l^{-1} ClH and to 8·00 with 1 mol l^{-1} NaOH. The effect of pH was tested after incubation at 25°C for 24 h (El-Nezami *et al.* 1998).

Stability AFB_1-bacteria complex

The stability of the bacteria-AFB_1 complex was evaluated by determining the amount of AFB_1 remaining bound after three washes. The AFB_1 stability assay was performed at pH 3·00, 5·00, 7·00 and 8·00 after incubation at 24 h. Bacterial pellets with bound AFB_1 were suspended in 2 ml of PBS (pH 7·00) and incubated for 10 min at 25°C. The bacteria were pelleted, and a volume of the supernatant was collected for the quantification of AFB_1 released from bacteria. This washing procedure was repeated another two times (Haskard *et al.* 2001; Peltonen *et al.* 2001).

Quantification of AFB_1 by HPLC

The quantification was performed by HPLC according to the methodology proposed by Trucksess *et al.* (1994) with some modifications (Cole and Dorner 1994), and the AFB_1 solutions (standards) were prepared according to AOAC (1995). An aliquot (200 μl) of the samples was derivatized with 700 μl trifluoroacetic acid: acetic acid: water (20 : 10 : 70, v/v). Chromatographic separations were performed on stainless steel, C18 reversed phase column (VARIAN, 150 × 4·6 mm id., 5 μm particle size). Water: methanol: acetonitrile (4 : 1 : 1, v/v) was used as mobile phase at a flow rate of 1·5 ml min^{-1}. The fluorescence of AFB_1 derivatives was recorded at excitation and emission wavelengths of 360 and 460 nm, respectively. The concentration of this toxin was quantified by correlating peak heights of sample extracts with those of standard curves. The detection limit of the analytical method was 0·1 ng g^{-1}. The percentage of mycotoxin bound to the bacteria was calculated using the equation: % Reduction = 100 × (1 − mycotoxin peak area of sample/mycotoxin peak area of control).

Statistical analysis

Data were analysed with an analysis of variance. Means were compared using a linear mixed model and Fisher's protected least significant difference (LSD) test to compare the AFB_1 binding along the treatments with AFB_1 in control assays. The analysis was conducted using software INFOSTAT, 2011 (Di Rienzo *et al.* 2011).

Results

Viability effect of *Enterococcus faecium* strains on AFB_1 binding

The viability of *Ent. faecium* cells after the preparation of two aliquots (aliquot 1: 1×10^8 CFU ml^{-1} of viable cells and aliquot 2: $10^8 \times 10$ CFU ml^{-1} of heat-killed cells) was determined by plate count method; 95% and 98% of *Ent. faecium* GJ40 and *Ent. faecium* MF4 cells in aliquot 1 remained viable, respectively. Both strains were 0% viable after aliquot 2 analyses (data not shown).

Figure 1 shows the effect of cell viability on AFB_1 binding capacity at pH 7·00 and 25°C. Results showed no significantly difference in AFB_1 removal between viable and nonviable cells in all treatments except for the interaction between *Ent. faecium* GJ40 at AFB_1 concentration of 50 ppb where the viable cells reduced the toxin concentration in a 20·55% and nonviable cell in a 15·45%, respect to the control ($P < 0·05$). The highest AFB_1 reduction was observed by *Ent. faecium* MF4 in the interaction assays using both toxin concentrations and both viable and nonviable cells; at 50 ppb viable cells reduced toxin concentration in 23·09% and nonviable cells in 25·51%. At 100 ppb, viable cells of *Ent. faecium* MF4 reduced AFB_1 in a 25·20% and nonviable cell in a 22·53%.

Incubation time effect on AFB_1 by *Enterococcus faecium* strains

Figure 2 shows the effect of incubation time on AFB_1 binding capacity by viable cells at pH 7·00 and 25°C. In general, the removal of AFB_1 slightly increased with extended incubation time, independently of concentration, for both strains; otherwise, results showed that *Ent. faecium* MF4 removed AFB_1 along the experiment with more efficiency than *Ent. faecium* GJ40 ($P < 0·05$). In the binding assay using 50 ppb of AFB_1 as control, *Ent. faecium* MF4 showed the highest reduction percentage (42·23%) at 48 h, while under the same conditions *Ent. faecium* GJ40 reduced the toxin concentration in a 27·35%. There were no statistical differences in the reduction of AFB_1 at 50 ppb by *Ent. faecium* GJ40 along the incubation time ($P < 0·05$). At 100 ppb, both strains reduced significantly AFB_1 respect to the control ($P < 0·05$), but in different percentage, being MF4 strain the most efficient (31·64% at 48 h).

pH effect on AFB_1 binding by *Enterococcus faecium* strains

The effect of pH on AFB_1 reduction by viable bacterial cells of *Ent. faecium* strains is shown in Table 1. Toxin

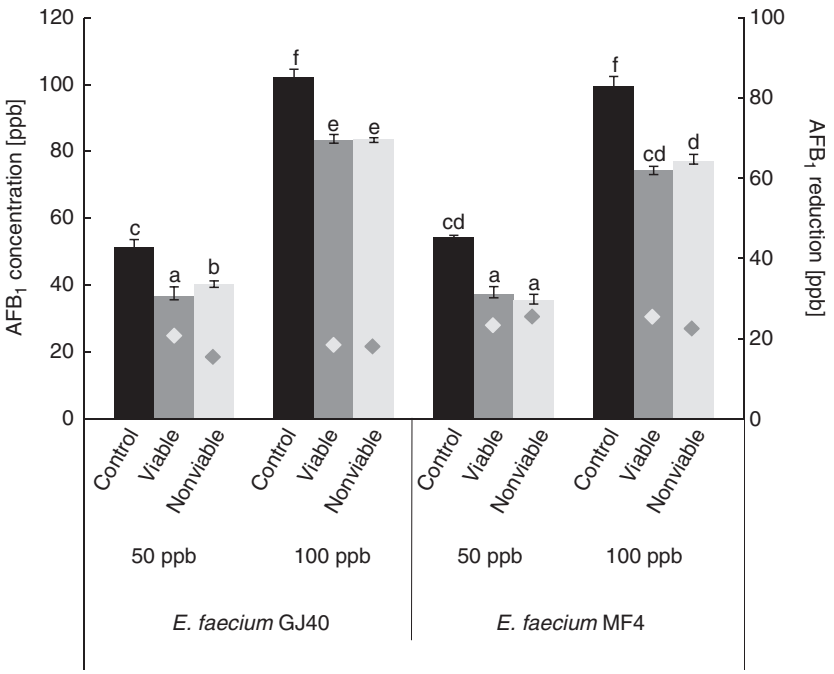

Figure 1 Aflatoxin B_1 detoxification capacity of *Enterococcus faecium* strains at pH 7·00 and 25°C. (●) % AFB_1 reduction by viable cells; (◆) % AFB_1 reduction by nonviable cells. SD: standard deviation. Values with the same letter are not significantly different according to least significant difference (LSD) test ($P < 0·05$). Statistical analysis compared means of the AFB_1 concentration of the phosphate buffered saline (PBS) as control and PBS with the toxin.

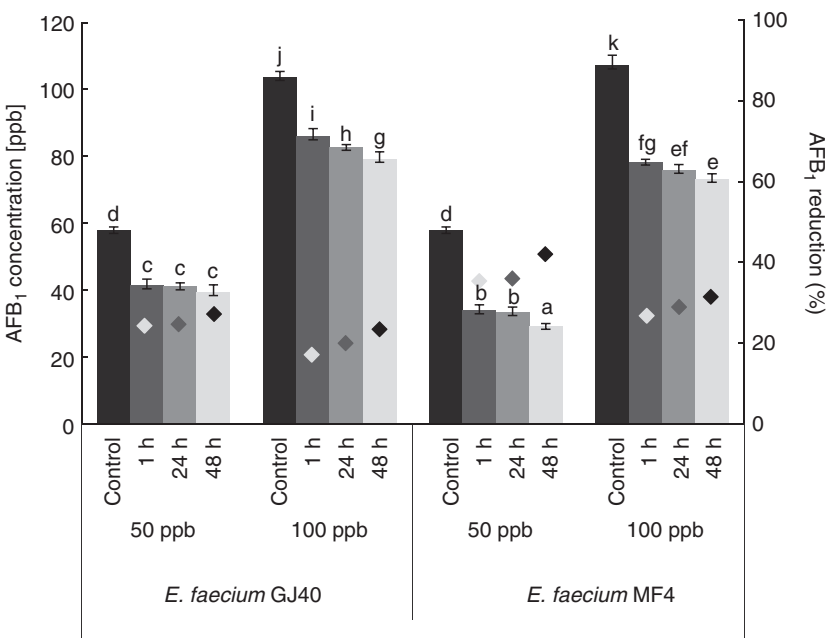

Figure 2 Effect of incubation time on AFB_1 detoxification by *Enterococcus faecium* viable cells at pH 7·00 and 25°C. (●) % AFB_1 reduction at 5 h; (◆) % AFB_1 reduction at 24 h; (◆) % AFB_1 reduction at 48 h. Values with the same letter are not significantly different according to least significant difference (LSD) test ($P < 0·05$). Statistical analysis compared means of the AFB_1 concentration of the phosphate buffered saline (PBS) as control and PBS with the toxin.

reduction by viable bacterial cells varied in the range of pH analysed for both AFB_1 concentrations used in the assay. The highest reduction percentage was obtained by *Ent. faecium* MF4 at pH 7·00 in the assay using 50 ppb (28·78%) and the lowest by *Ent. faecium* GJ40 at pH 8·00 using 100 ppb (10·75%). In general, AFB_1 removal was major at pH 7 for both concentrations assayed by both *Ent. faecium* strains. No significant differences in AFB_1 binding capacity by *Ent. faecium* strains were detected. Significant differences were found between the concentrations assayed ($P < 0·05$).

Binding stability of bacteria-AFB_1 complex

Figure 3 shows the stability of the bacteria-AFB_1 complex after three washes with PBS at pH 7·00. Aflatoxin B_1 still

Table 1 Effect of pH on AFB_1 detoxification by *Enterococcus faecium* strains isolated from healthy dog faeces at 25°C for 24 h

	Ent. faecium GJ40				*Ent. faecium* MF4			
	50 ppb		100 ppb		50 ppb		100 ppb	
PBS*	(AFB_1)* ± SD	AFB_1 (%)†	(AFB_1)* ± SD	AFB_1 (%)†	(AFB_1)* ± SD	AFB_1 (%)†	(AFB_1)* ± SD	AFB_1 (%)†
pH 3·00 + AFB_1	$54·06 \pm 2·00^{efg}$	**$18·49^{e}$**	$101·9 \pm 2·2^{lm}$	**$14·46^{c}$**	$52·23 \pm 3·48^{def}$	**$26·80^{j}$**	$101·13 \pm 1·20^{lm}$	**$20·56^{f}$**
pH 3·00 + AFB_1 + bacteria	$44·23 \pm 1·05^{bc}$		$87·16 \pm 1·75^{ij}$		$38·23 \pm 0·45^{ab}$		$80·33 \pm 0·51^{hlm}$	
pH 5·00 + AFB_1	$57·06 \pm 1·93^{g}$	**$15·65^{cd}$**	$104·86 \pm 4·47^{m}$	**$10·90^{a}$**	$58·13 \pm 1·95^{g}$	**$24·47^{i}$**	$103·16 \pm 2·15^{lm}$	**$12·69^{b}$**
pH 5·00 + AFB_1 + bacteria	$48·13 \pm 0·80^{cd}$		$93·43 \pm 4·3^{k}$		$43·90 \pm 1·25^{ab}$		$90·06 \pm 0·80^{jk}$	
pH 7·00 + AFB_1	$52·46 \pm 2·45^{ef}$	**$24·77^{i}$**	$101·62 \pm 2·45^{lm}$	**$20·22^{f}$**	$56·13 \pm 2·45^{fg}$	**$28·78^{k}$**	$101·6 \pm 1·74^{lm}$	**$21·06^{g}$**
pH 7·00 + AFB_1 + bacteria	$39·46 \pm 0·83^{a}$		$81·06 \pm 0·83^{h}$		$40·00 \pm 0·83^{ab}$		$80·20 \pm 1·05^{h}$	
8·00 + AFB_1	$57·96 \pm 2·00^{g}$	**$10·75^{a}$**	$99·63 \pm 8·71^{l}$	**$16·66^{d}$**	$56·76 \pm 3·97^{g}$	**$22·25^{gh}$**	$102·96 \pm 3·70^{lm}$	**$22·79^{h}$**
pH 8·00 + AFB_1 + bacteria	$51·73 \pm 1·00^{de}$		$83·03 \pm 2·02^{hi}$		$44·13 \pm 1·42^{bc}$		$79·50 \pm 0·81^{h}$	

PBS, phosphate buffer solution adjusted at different pH values; SD, standard deviation.
Mean values based on triplicated data. Values with the same letter are not significantly different according to least significant difference test ($P < 0·05$).
Statistical analysis was performed separately for concentration and percentage values and statistical data must be red separately.
*Concentration of AFB_1.
†Percentage of AFB_1 reduction.

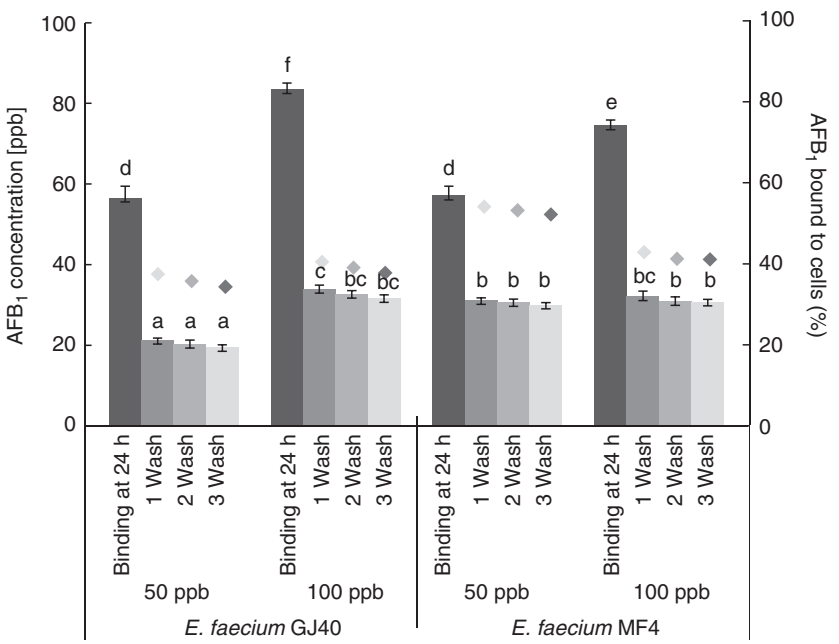

Figure 3 Stability of the complex viable bacteria-AFB_1 after three washes with phosphate buffered saline (PBS) at pH 7·00 after 24 h. (●) % AFB_1 bound at one wash; (◆) % AFB_1 bound at two wash; (◆) % AFB_1 bound at three wash. Values with the same letter are not significantly different according to least significant difference (LSD) test ($P < 0·05$). Statistical analysis compared means of the AFB_1 concentration in the binding at 24 h and AFB_1 concentration remained in the cell after the washing procedure.

remains bound in the cell of *Ent. faecium* GJ40 in a 34–37% and in 41·31–52·62% in *Ent. faecium* MF4 after three washings with PBS. In all cases, no significant differences in the release of the toxin were found after the first wash ($P < 0·05$). The pH of washing solution (3·00, 5·00 and 8·00) did not affect the binding stability of bacteria-AFB_1 complex (data not shown).

The LSD test of data shows the significant differences among control treatments and interacting assays. The statistical analysis of strains showed that all analysed factors influenced significantly on AFB_1 binding ($P < 0·05$) (Table 2).

Discussion

The present study shows the capacity of removing 50 and 100 ppb of AFB_1 in aqueous solution by two *Ent. faecium* strains isolated from healthy dog faeces. Viable and non-viable cells of both strains (GJ40 and MF4) removed AFB_1 in different percentages along the experiments, but

Table 2 Analysis of variance of effect of strains (S), AFB_1 concentration (C), viability (V), incubation time (I) pH (P) and their interactions on AFB_1 binding

		AFB_1 binding	
Source of variation	df	MS	F
S	1	94·74	23·99*
C	1	5319·27	1347·03*
V	2	1342·54	339·98*
I	3	1751·59	610·98*
P	3	158·54	23·52*
S × C × V	2	11·60	2·94*
S × C × I	3	3·00	1·05*
S × C × P	3	2·41	0·36*

df, degrees of freedom; MS, mean square, F, F-Snedecor.
*Significant $P < 0·05$.

Ent. faecium MF4 was more effective in the removal of the toxin. The results obtained are similar of those obtained by Topcu *et al.* (2010), who assayed the binding capacity of two probiotic *Ent. faecium* strains. The researchers found that the strains were able to bind AFB_1 in high percentages under different conditions of pH, viability and incubation time (46%).

In the viability assay, no significant differences were found in AFB_1 binding by viable and nonviable *Ent. faecium* GJ40, but the toxin removal by *Ent. faecium* MF4 viable was significantly different respect to the nonviable cells, even though the reduction percentage was similar. According to this, it can be considered that detoxification of AFB_1 by *Ent. faecium* may be due to the binding of the mycotoxin to the bacterial cell wall components. This mechanism has been studied and also postulated by other authors (El-Nezami *et al.* 1998; Haskard *et al.* 2000, 2001). The cell wall peptidoglycans and polysaccharides have been proposed to be responsible for mycotoxin binding by bacteria (Haskard *et al.* 2001; Lahtinen *et al.* 2004). In several previous studies, it has been reported that heat treated bacteria were more effective in removing AFB_1 than viable cells (Shetty and Jespersen 2006; Topcu and Bulat 2010); the results obtained in the present work are similar to another studies reporting that no significant differences were found in the removal of the toxin by viable and nonviable cells (El-Nezami *et al.* 1998; Haskard *et al.* 2001; Pizzolito *et al.* 2011). Therefore, the fact that nonviable and viable micro-organisms are able to remove AFB_1 *in vitro* conditions with similar efficiency suggests that the removal process does not require metabolic conversion of the toxin by cells and it could be dependent on the strain. Additionally, these results indicate that the inclusion of viable or nonviable micro-organisms in the diet of animals would be equally effective against aflatoxicosis. This is very important because the use of nonviable cells decreases the potential pathogenic risks of their inclusion in the diet. Besides, the use of viable cells is an interesting point as the assayed *Ent. faecium* strains have benefic properties for the host, such as antimicrobial activity (Fernández Juri *et al.* 2013).

About the effect of incubation time, Peltonen *et al.* (2001) reported that the removal of AFB_1 was a fast process and the AFB_1 binding by *Lactobacillus amylovorus* CSCC 5197 increased from 52 (0 h) to 68% (48 h). In another study, it was observed that at cero hour the percentage of removed AFB_1 was not significantly different from the one at 72 h (Pizzolito *et al.* 2011). On the other hand, Topcu *et al.* (2010) reported that at 1 h, *Ent. faecium* M74 and EF031 strains removed AFB_1 which was approximately 65% of the total of AFB_1 removed throughout the whole incubation period of 48 h. In the present work, both strains at both concentrations detoxificated AFB_1 quickly at 1 h, but the removal maintained similar up to 48 h of assay, even though there were significant differences. These results suggest that the binding of the toxin occurs quickly, but does not increase the removal considerably with the incubation time.

Regarding to pH effect, the highest reduction percentage was obtained by *Ent. faecium* GJ40 at pH 7·00. Respect to *Ent. faecium* MF4, pH values assayed (3·00, 5·00, 7·00 and 8·00) did not affect statistically the removal of the toxin ($P < 0·05$), except at pH 5 using 100 ppb. These results partially agree with those previously reported. Some researchers reported that binding process is not exclusively dependent of pH. Zinedine *et al.* (2005) reported that all the *Lactobacillus* spp. assayed removed the toxin from 5% to 40% when pH increased from 3 to 5·5. On the other hand, Rayes (2013) reported that the highest reduction percentage of AFB_1 by a pool of probiotic LAB occurred pH 8·5 while the lowest removal was at pH 4·5. When the pool was combined with a *Saccharomyces cerevisiae* strain, the highest removal was at pH 4·5 and the lowest removal was at pH 8·5. In contrast, results in the binding capacity assay using *Lactobacillus rhamnosus* strains at pH 3 and 6 showed that there were no significant differences in the toxin reduction between any bacterial cell conditions (in solution, spray or freeze-dried) (Bovo *et al.* 2014). Similarly, Peltonen *et al.* (2001) assayed 12 *Lactobacillus*, five *Bifidobacterium* and three *Lactococcus* strains and concluded that the differences in AFB_1 binding by the strains were probably due to different bacterial cell wall and cell envelope structures. So, it can be said that the pH and incubation time dependence of AFB_1 binding vary between bacterial strains and their cell wall components.

Respecting the toxin concentration effect, Rahayu *et al.* (2007) demonstrated that increasing AFB_1 concentration

in aqueous medium did not affect the percentage of AFB_1 binding; however, it affects the binding speed. In addition, Pizzolito *et al.* (2011), in a binding assay with yeast and LAB using different AFB_1 concentrations, reported that the removal of the toxin depended of the strain; some were more effective at a lower concentration (*Lact. rhamnosus* I at 50 ppb) and some others were more effective at a higher concentration (*Lactobacillus acidophilus* 24 at 100 ppb and *Lactobacillus casei* subsp. *rhamnosus* at 500 ppb). The results obtained in the present work are similar to the mentioned above, as *Ent. faecium* strains were able to bind AFB_1 in higher percentages in the assay using 50 ppb of AFB_1, but this ability depends on the strain.

Related to stability of the viable cell-AFB_1 complex, results suggest that binding is a fast and reversible process. On the other hand, it could be proposed that metabolic conversion of the toxin by cells did not take place, because AFB_1 was released in the same chemical form from bacterial cells. Several researchers have previously reported the reversibility of the process; Haskard *et al.* (2001) analysed 12 LAB strains in both viable and nonviable forms, exhibiting reversible binding of AFB_1 after five washing steps with water (6–11%). Hernandez-Mendoza *et al.* (2009) reported that using PBS solution, about 60–70% of AFB_1 remained bound to the cells, suggesting that the toxin is attached to the bacteria by weak, no covalent interactions that could be at least partially reversible. Pizzolito *et al.* (2011) reported that after five washings with PBS, AFB_1 bound to different LAB cells were close to 50%, and the washing time did not change the release percentages when it varied from 1 to 60 min. Similarly, the results obtained in the present work showed that the toxin remained in the bacterial cells in a high percentage (35–50%) after three washes with PBS solution. According to this, it could be suggested that neither the entrance of AFB_1 into the cell nor its metabolic conversion is necessary; therefore, micro-organism cell wall components may be involved in AFB_1 removal, as was suggested by several authors (Lahtinen *et al.* 2004; Karaman *et al.* 2005). These results may be attributed to differences of cell wall structure characteristics of each strain.

Concluding, the results obtained in the present work show that *Ent. faecium* GJ40 and *Ent. faecium* MF4 assayed possess the capacity of removing AFB_1 from aqueous solutions with different efficiency under the assayed conditions. Even though the reduction percentages of AFB_1 in general were below 30%, these strains could be used as additives in dry pet food naturally contaminated with low levels of this toxin (Campos *et al.* 2009; Fernández Juri *et al.* 2009b). In addition, these strains also present benefic properties previously assayed (Fernández Juri *et al.* 2013). Further experiments simulating the intestinal conditions are needed to determine the efficiency of toxin removal and once the bacteria are added to the pet food.

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Conflict of Interest

The authors of this manuscript declare that they have no conflict of interest.

References

Aquino, S. and Corrêa, B. (2011) Aflatoxins in pet foods: a risk to special consumers. In *Aflatoxins – Detection, Measurement and Control* ed. Torres-Pacheco, I. pp. 53–74. Croatia: InTech, ISBN: 978-953-307-711-6.

Association of Official Analytical Chemists (A.O.A.C) (1995) *Official Methods of Analysis*, 16th edn. Arlington, Virginia: AOAC. International.

Baptista, A.S., Horii, J., Calori-Domingues, M.A., da Gloria, E.M., Salgado, J.M. and Vizioli, M.R. (2004) The capacity of manno-oligosaccharides, thermolysed yeast and active yeast to attenuate aflatoxicosis. *World J Microbiol Biotechnol* **20**, 475–481.

Basalan, M., Hismiogullari, S.E., Hismiogullari, A.A. and Filazi, A. (2004) Fungi and aflatoxin B_1 in horse and dog feeds in Western Turkey. *Revue Méd Vet* **156**, 248–252.

Binder, E.M. (2006) Managing the risk of mycotoxins in modern feed production. *Anim Feed Sci Technol* **133**, 149–166.

Boermans, H.J. and Leung, M.C.K. (2007) Mycotoxins and the pet food industry: toxicological evidence and risk assessment. *Int J Food Microbiol* **19**, 95–102.

Bolognani, F., Rumney, C.J. and Rowland, I.R. (1997) Influence of carcinogen binding by lactic acid-producing bacteria on tissue distribution and *in vitro* mutagenecity of dietary carcinogens. *Food Chem Toxicol* **35**, 535–545.

Bovo, F., Franco, L.T., Rosim, R.E., Fávaro Trindade, C.S. and Fernandes de Oliveira, C.A. (2014) The ability of *Lactobacillus rhamnosus* in solution, spray-dried or lyophilized to bind aflatoxin B_1. *J Food Res* **3**, 35.

Campos, G.S., Cavaglieri, L.R., Fernández-Juri, M.G., Kruger, C., Dalcero, A.M., Magnoli, C. and Rosa, C.A.R. (2008)

Mycoflora and aflatoxins in raw materials and pet food in Brazil. *J Anim Physiol Anim Nutr* **92**, 377–383.

Campos, S.G., Keller, L.M., Cavaglieri, L.R., Kruger, C., Fernández Juri, M.G., Dalcero, A.M., Magnoli, C.E. and Rosa, C.A.R. (2009) Aflatoxigenic fungi and aflatoxins in commercial ready pet food in Brazil. *World Mycotox J* **2**, 85–90.

CAST Report (2003) Chapter 2: Introduction. In *Mycotoxins: Risks in Plant, Animal, and Human Systems* ed. Richard, J.L. and Payne, G.A. Council for Agricultural Science and Technology Task Force report No. 139, Ames, Iowa, USA.

Celyk, K., Denly, M. and Savas, T. (2003) Reduction of toxic effects of aflatoxin by using Baker yeast (*Saccharomyces cerevisiae*) in growing broiler chicken diets. *Rev Bras Zootec* **32**, 615–619.

Cole, R.J. and Dorner, J.W. (1994) Extraction of Aflatoxins from naturally contaminated peanuts with different solvents and solvent peanut ratios. *J AOAC Int* **77**, 1509–1511.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M. and Robledo, C.W. (2011) InfoStat versión 2011. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina. Available at: http://www.infostat.com.ar.

El-Nezami, H., Kankaanpaa, P., Salminen, S. and Ahokas, J. (1998) Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, aflatoxin B_1. *Food Chem Toxicol* **36**, 321–326.

El-Nezami, H.S., Chrevatidis, A., Auriola, S., Salminen, S. and Mykkänen, H. (2002) Removal of common *Fusarium* toxins *in vitro* by strains of *Lactobacillus* and *Propionibacterium*. *Food Addit Contam* **19**, 680–686.

Fernández Juri, M.G., Bressán, F., Astoreca, A.L., Barberis, C.L., Campos, G.S., Cavaglieri, L.R., Dalcero, A.M. and Magnoli, C.E. (2009a) Aflatoxins, fumonisins and toxigenic fungi in raw materials and ready dry dog food in Argentina. *Rev Bras Med Vet* **31**, 109–117.

Fernández Juri, M.G., Bressán, F., Astoreca, A.L., Barberis, C.L., Campos, G.S., Cavaglieri, L.R., Dalcero, A.M. and Magnoli, C.E. (2009b) Mycotoxicological quality of different commercial extruded dog food in Argentina. *Rev Bras Med Vet* **31**, 272–281.

Fernández Juri, M.G., Muzzolón, J.A., Dalcero, A.M. and Magnoli, C.E. (2011) Effect of acid lactic bacteria isolated from faeces of healthy dogs on growth parameters and aflatoxin B_1 production by *Aspergillus* species *in vitro*. *Mycotoxin Res* **27**, 273–280.

Fernández Juri, M.G., Muzzolón, J., Dalcero, A.M. and Magnoli, C.E. (2013) Inhibitory properties of *Enterococcus* spp. isolated from faeces of healthy dogs. *J Environ Sci Health, Part B* **48**, 983–992.

Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M. and Knasmuller, S. (2008) Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. *Food Chem Toxicol* **46**, 1398–1407.

Giraffa, G. (2003) Functionality of enterococci in dairy products. *Int J Food Microbiol* **88**, 215–222.

Haskard, C., Binnion, C. and Ahokas, J. (2000) Factors affecting the sequestration of aflatoxin by *Lactobacillus rhamnosus* strain GG. *Chem Biol Interact* **128**, 39–49.

Haskard, C.A., El-Nezami, H.S., Kankaanpää, P.E., Salminen, S. and Ahokas, J.T. (2001) Surface binding of aflatoxin B_1 by lactic acid bacteria. *Appl Environ Microbiol* **67**, 3086–3091.

Hernandez-Mendoza, A., Garcia, H.S. and Steele, J.L. (2009) Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B_1. *Food Chem Toxicol* **47**, 1064–1068.

Juodeikiene, G., Basinskiene, L., Bartkiene, E. and Matusevicius, P. (2012) Mycotoxin decontamination aspects in food, feed and renewables using fermentation processes. In *structure and function of food engineering* ed. Amer Eissa, A. ISBN: 978-953-51-0695-1. doi: 10.5772/46184.

Karaman, M., Basmacioglu, H., Ortatatli, M. and Oguz, H. (2005) Evaluation of the detoxifying effect of yeast glucomannan on aflatoxicosis in broilers as assessed by gross examination and histopathology. *Br Poult Sci* **46**, 394–400.

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

Leung, M.C.K., Díaz-Llano, G. and Smith, T.K. (2006) Mycotoxins in pet food: a review on worldwide prevalence and preventative strategies. *J Agric Food Chem* **54**, 9623–9635.

Park, D.L. (1993) Perspectives on mycotoxin decontamination procedures. *Food Addit Contam* **10**, 49–60.

Peltonen, K., El-Nezami, H., Haskard, C., Ahokas, J. and Salminen, S. (2001) Aflatoxin B_1 binding by dairy strains of lactic acid bacteria and bifidobacteria. *J Dairy Sci* **84**, 2152–2156.

Pizzolito, R.P., Bueno, D.J., Armando, M.R., Cavaglieri, L., Dalcero, A.M. and 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. In *Aflatoxins-Biochemistry and Molecular Biology* ed. Guevara-Gonzalez, R.G. ISBN: 978-953-307-395-8. doi: 10.5772/23717.

Rahayu, E.S., Amanah, H.Z., Utami, R. and Utami, T. (2007) Study on binding of AFB_1 by *Lactobacillus acidophilus* SNP-2. Asian Conference of Lactic Acid Bacteria 2007, Shianghai, China.

Rayes, A.A.H. (2013) Removal of aflatoxin B_1 from experimentally contaminated whole milk using a pool of probiotic strains of lactic acid bacteria and baker's yeast *Saccharomyces* cerevisiae. *N Y Sci J* **6**, 84–90.

Santin, E., Paulilo, A.C., Maiorka, A., Nakaghi, L.S.O., Macan, M. and de Silva, A.V.F. (2003) Evaluation of the efficiency

of *Saccharomyces cerevisiae* cell wall to ameliorate the toxic effects of aflatoxin in broilers. *Int J Poult Sci* **2**, 241–344.

Santos, A., Marquina, D., Leal, J.A. and Peinado, J.M. (2006) (1-6)-β-D-glucan as cell wall receptor for *Pichia membranifaciens* killer toxin. *Appl Environ Microbiol* **66**, 1809–1813.

Shetty, P. and Jespersen, L. (2006) *Saccharomyces cerevisiae* and lactic acid bacteria as potential mycotoxin decontaminating agents. *Trends Food Sci Technol* **17**, 48–55.

Simonetta, A.C., Moragues de Velasco, L.G. and Frison, L.N. (1997) Antibacterial activity of Enterococci strains against *Vibrio cholerae*. *Lett Appl Microbiol* **24**, 139–143.

Suchodolski, J.S., Ruaux, C.G., Steiner, J.M., Fetz, K. and Williams, D.A. (2004) Application of molecular fingerprinting for qualitative assessment of small-intestinal bacterial diversity in dogs. *J Clin Microbiol* **42**, 4702–4708.

Topcu, A. and Bulat, T. (2010) Removal of cadmium and lead from aqueous solution by *Enterococcus faecium* strains. *J Food Sci* **75**, 13–17.

Topcu, A., Bulat, T., Wishah, R. and Boyaci, I.H. (2010) Detoxification of aflatoxin B_1 and patulin by *Enterococcus faecium* strains. *Int J Food Microbiol* **139**, 202–205.

Trucksess, M.W., Stack, M.E., Nesheim, S., Albert, R. and Romer, T. (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. *J AOAC Int* **77**, 1512–1521.

Zinedine, A., Faid, M. and Benlemlih, M. (2005) *In vitro* reduction of aflatoxin. *Int J Agric Biol* **7**, 67–70.