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## **An *in vitro* Investigation of Aflatoxin B<sub>1</sub> Biological Control by *Lactobacillus plantarum***

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**Abstract:** This study assessed the binding of Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) from contaminated solution by *Lactobacillus plantarum* PTCC 1058. This strain and AFB<sub>1</sub> was incubated (1, 24, 48, 90 h at 37°C) and the amount of unbound AFB<sub>1</sub> was quantities by HPLC. The concentration of AFB<sub>1</sub> in solution was 0.5 ppm. The stabilities of the bacteria/AFB<sub>1</sub> complexes were evaluated by determining the amount of AFB<sub>1</sub> remaining bound following three washes. Effect of Incubation time on AFB<sub>1</sub> Binding on viable and dead cells were evaluated at 1, 24, 48, 72 and 90 h time points. In 1 h 45% and in 90 h 100% AFB<sub>1</sub> was removed from solution by this strain. Autoclaved bacteria didn't remove AFB<sub>1</sub> from solutions efficiently (31% in 1 h and 15% in 24 h). Bacteria in logarithmic growth phase retained 92% of the AFB<sub>1</sub> initially bound after three washes. Bacterial binding of AFB<sub>1</sub> by this strain was rapid and they were in logarithmic growth phase. These findings further support the ability of specific strains of lactic acid bacteria to bind selected dietary contaminants.

**Key words:** *Lactobacillus plantarum*, AFB<sub>1</sub>, HPLC

### **INTRODUCTION**

Aflatoxins are a group of mycotoxins with mutagenic, carcinogenic and immunosuppressive properties (Eaton and Gallagher, 1994). The occurrence of aflatoxin contamination is global, with severe problems especially prevalent in developing countries (Henry *et al.*, 1999). They are synthesized as secondary metabolites of toxigenic *Aspergillus flavus*, *Aspergillus paraciticus* and *Aspergillus nomius* strains. *Aspergillus flavus* only produces aflatoxin B<sub>1</sub> and B<sub>2</sub> and *Aspergillus paraciticus* produces these same metabolites along with G<sub>1</sub> and G<sub>2</sub>. *Aspergillus flavus*, is missing the critical piece of DNA that converts a precursor to aflatoxin G<sub>1</sub>, so it is the reason why *Aspergillus flavus* makes only aflatoxin B<sub>1</sub> and B<sub>2</sub> (Ehrlich *et al.*, 2004). When aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and B<sub>2</sub> contaminated food or feed is consumed, the toxins are metabolized to aflatoxins M<sub>1</sub> and M<sub>2</sub> and excreted into the tissues, biological fluids and milk of lactating animals, including breast milk (Zarba *et al.*, 1992). AFB<sub>1</sub> affects liver causing cirrhosis, hepatoma, hepatitis and Rey's disease as well as affect other organs like kidney, myocardium and muscles. Even it may lead to decreased immunity in animal (Fernandez *et al.*, 2000).

These fungi grow on a variety of food and feed commodities at any stage during growth, harvest, storage and transportation. Aflatoxins are also of industrial

importance due to the economic losses resulting from condemnation of contaminated crops, cheese defects and impaired growth and feed efficiency of animals fed contaminated feeds (Haskard *et al.*, 2001).

El-Nezami studies have shown that two probiotic strains, *Lactobacillus rhamnosus* strain GG (ATCC 53103) and *L. rhamnosus* strain LC-705 (DSM 7061); efficiently remove AFB<sub>1</sub> from solution (El-Nezami *et al.*, 1998a, b). Lactic acid bacteria are used in dairy product and fermentation process as starter culture. Their main role is organic acid production and their use as bio preservative (Haskard *et al.*, 2001; El-Nezami *et al.*, 1996).

The objectives of this study were find native lactic acid bacteria strain [*Lactobacillus plantarum* (PTCC 1058)] that efficiently bind aflatoxin B<sub>1</sub>, examine the stability of the bacteria/AFB<sub>1</sub> complexes formed and study the effect of an extended incubation time on toxin binding.

### **MATERIALS AND METHODS**

All data reported in this study are from triplicate measurement.

**Bacterial strains, culture conditions and estimation of bacterial concentration:** *Lactobacillus plantarum* (PTCC 1058) was studied for biocontrol of aflatoxin B<sub>1</sub>. The strain was originally obtained from Dr. Mohseni at Persian Type

Culture Collection (PTCC) of Iranian Research Organization for science and Technology in Tehran-Iran. Bacterial strain was cultivated in de Mann, Rogosa, Sharpe broth (MRS, Hi-media, India) for 24 h at 37°C in a 5% CO<sub>2</sub>/95% air atmosphere was followed according to El-Nezami *et al.* (1998a). Bacteria concentration in culture flask was performed using spectrophotometry assay in 600 nm to obtain a bacteria concentration a round  $1 \times 10^9$  cfu mL<sup>-1</sup>. Culture broth of MRS was collected to determine the logarithmic growth phase of *Lb. plantarum* (PTCC 1058). The absorbance was measured at 600 nm by a spectrophotometer Unicam 5625 UV/VIS, every 2 h.

**Aflatoxin binding assay:** Modified AFB<sub>1</sub> binding assay was employed (Peltonen, 2001). Briefly, AFB<sub>1</sub> (Sigma, St. Louis, MO.) was dissolved in methanol and the concentration was determined at 348 nm by spectrophotometer ( $\epsilon_{348} = 19,800 \text{ M}^{-1} \text{ cm}^{-1}$ ). A solution of 0.5 µg mL<sup>-1</sup> AFB<sub>1</sub> was prepared in PBS (pH 7/3, 0.01 M) and the methanol was evaporated by heating in a water bath (70°C, 5 to 10 min). Bacterial samples were centrifuged at 2500×g for 12 min and the bacterial pellets were washed with PBS. The supernatant was removed prior to AFB<sub>1</sub> binding assays, incubations were carried out at 37°C (El-Nezami *et al.*, 1998b). The bacterial pellet was suspended in PBS (1.5 mL, pH 7/3, 0.01 M) containing 0.5 µg of AFB<sub>1</sub> (500 ng) per mL and incubated at 37°C for 1, 24, 48, 72 and 90 h and centrifuged prior to analysis by either high performance liquid chromatography (HPLC). All assays were performed in triplicate, a both positive control (PBS substituted for bacteria) and a negative control (PBS substituted for AFB<sub>1</sub>) was also incubated.

**HPLC assay:** Reverse-phase HPLC was used to quantify AFB<sub>1</sub> remaining in the supernatant of bacteria incubated with AFB<sub>1</sub>. The HPLC system (Applied Bio systems) was fitted with a UV detector and an ODS Spheri-5 Brownlee column (250 by 4.6 mm, 5 µm) fitted with a C<sub>18</sub> guard column. Deionised water/acetonitrile/methanol (58:21:21, vol/vol/vol) was used as the mobile phase, with a flow rate of 1.25 mL min<sup>-1</sup>. The assay was carried out at room temperature with an injection volume of 70 µL. Detection was done by UV with wavelength of 365nm. The retention time was 15 min (Manda *et al.*, 2004).

Standard AFB<sub>1</sub> solutions with different concentrations, 50, 100, 200, 500, 1000 and 5000 ppb (ng mL<sup>-1</sup>) were prepared to determine the calibration curve of HPLC system with UV detector.

The percentage of AFB<sub>1</sub> bound to the bacteria was calculated using the formula according to Peltonen *et al.* (2001).

$$100\% \times (1 - \frac{\text{AFB}_1 \text{ peak area of sample}}{\text{peak area of } 0.5 \mu\text{g mL}^{-1} \text{ control}})$$

**Bacteria/AFB<sub>1</sub> complex stability:** The stabilities of the bacteria/AFB<sub>1</sub> complexes were evaluated by determining the amount of AFB<sub>1</sub> remaining bound following three washes. Bacterial pellet was washed by being suspended in 1.5 mL PBS (pH 7.3, 0.01 M) and incubated at 37°C for 10 min according to El-Nezami *et al.* (1998a).

**Effect of incubation time on afb<sub>1</sub> binding:** Bacterium ( $1 \times 10^9$  cfu mL<sup>-1</sup>) and AFB<sub>1</sub> (1.5 mL, 0.5 µg mL<sup>-1</sup>) was incubated at 37°C for 90 h and supernatant samples (200 µL) were collected after centrifugation (2500×g for 12 min) at 1, 24, 48, 72 and 90 h time points. The supernatant was removed, released AFB<sub>1</sub> was quantified by HPLC and the percent AFB<sub>1</sub> bound was calculated.

**Removal of aflatoxin by autoclaved cells:** Incubation of  $1 \times 10^9$  cfu mL<sup>-1</sup> dead cells (autoclaved at 121°C and 1.4 atm pressure for 20 min) per mL with AFB<sub>1</sub> (1.5 mL, 0.5 µg mL<sup>-1</sup>) during 1, 24, 48, 72 and 90 h at 37°C. After centrifugation at 2500×g for 12 min, the supernatant was removed, released AFB<sub>1</sub> was quantified by HPLC and the percent AFB<sub>1</sub> bound was calculated.

**Statistical analysis:** Significant differences between bacterial treatments were tested by analysis of variance using Minitab. Data were normalized and Tukey tests were performed. The results of different incubated time experiments were subjected to Student's t-test to identify significant differences between bacterial treatments. Probability (p) values of <0.05 were considered significant.

## RESULTS

The amount of AFB<sub>1</sub> bound to *Lb. plantarum* (PTCC 1058) was studied exhibited a distribution of AFB<sub>1</sub> binding properties (Fig. 1). The AFB<sub>1</sub> binding of *Lb. plantarum* (PTCC 1058) was increased significantly ( $p < 0.05$ ) with extended incubation time from 45 (1 h) to 100% (90 h) (Fig. 1).

HPLC with UV detector standard curve demonstrated the 0.99 correlation coefficient between concentration of AFB<sub>1</sub> and its peak area in chromatograms.

The results of bacteria growth curve revealed that *Lb. plantarum* (PTCC 1058) after 10 h entered in logarithmic growth phase and this stage took 72 h and after it the stationary phase began and continued for another 168 h and finally entered in dead phase.

When washing the *Lb. plantarum*/AFB<sub>1</sub> complexes, bacteria in logarithmic growth phase retained 92% of the AFB<sub>1</sub> initially bound (Table 1). The release of AFB<sub>1</sub> was lowest during the first wash and it was nearly to 0% in third wash.

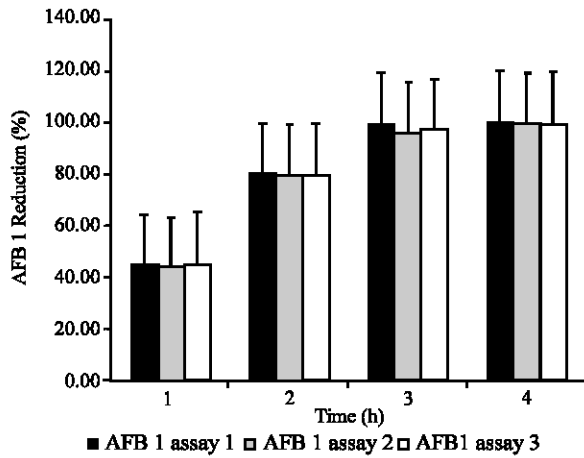


Fig. 1: Amount of AFB<sub>1</sub> absorption by *Lb. plantarum* (PTCC 1058) after 1-90 h period of incubation time in triplicate assays. The most amount of reduction (100%) was after 90 h ( $p < 0.05$ )

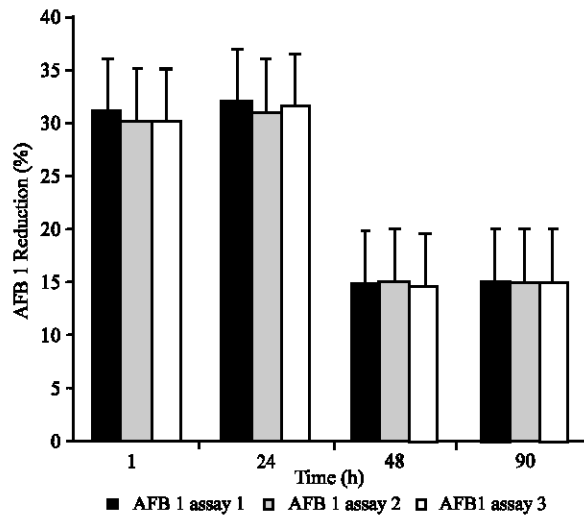


Fig. 2: Effect of autoclaved bacteria in AFB<sub>1</sub> reduction. Autoclaved *Lb. plantarum* (PTCC 1058) reduced only 31% of AFB<sub>1</sub> of solutions in 1, 24 h and 15% of it after 48, 90 h incubation ( $p < 0.05$ )

Table 1: Effect of washes on AFB<sub>1</sub>/*Lactobacillus* complex stability

(% AFB <sub>1</sub> released back (SD) Strain	Washes		
	1	2	3
<i>Lb. plantarum</i> (PTCC 1058)	8.7	0.14	0

Each value is a mean ± (standard deviation) of triplicate assays

Autoclaved bacteria didn't remove AFB<sub>1</sub> from solutions efficiently and by increasing incubation time to 90 h this value decreased (Fig. 2).

There was changed in bacterial shape after 90 h treatment with 0.5 ppm AFB<sub>1</sub> solutions by gram staining.

They became shorter in rod length and their gram positive characteristic changed to gram negative.

## DISCUSSION

*Lb. plantarum* (PTCC 1058) tested in this study was more efficient in binding AFB<sub>1</sub> than the reported previously, by Haskard *et al.* (2001). The complexes formed between AFB<sub>1</sub> and *Lb. plantarum* (PTCC 1058) was also significantly more stable than those formed with the other strains tested by Haskard *et al.* (2001) ( $p < 0.05$ ). El-Nezami *et al.* (1996, 1998a, 2000) reported that specific dairy strains of Lactobacilli can remove aflatoxins from aqueous solution. In addition, specific dairy strains of lactic acid bacteria also removed aflatoxin M<sub>1</sub> from reconstituted milk Pierides *et al.* (2000). The removal of aflatoxin involves physical binding of the toxin probably to the bacterial cell wall or cell wall components (El-Nezami *et al.*, 1998b; Haskard *et al.*, 2001). The ability of *Lb. plantarum* (PTCC 1058) to eliminate AFB<sub>1</sub> from the media was demonstrated in this study. Differences in the removal of AFB<sub>1</sub> was noted. The removal of AFB<sub>1</sub> was dependent on incubating time. *Lb. plantarum* removed 45% of AFB<sub>1</sub> after 1h and by increasing incubation time it could reduce AFB<sub>1</sub> efficiently from solution (100%). Theoretical calculations by Oatley *et al.* (2000) demonstrate that AFB<sub>1</sub> removal does not arise solely from trapping of the toxin in the bacterial pellet during centrifugation. Metabolic conversion and covalent binding of AFB<sub>1</sub> by the bacteria have been excluded as a mechanism of removal and noncovalent binding of AFB<sub>1</sub> to the bacteria has been proposed (Zhang *et al.*, 1993). The effect of heat treatment was examined on the aflatoxin removal process. The heat treatment bacteria was found to markedly decrease the bacterial AFB<sub>1</sub> binding ability, although Haskard *et al.* (2001) demonstrated that heat treated bacteria removed AFB<sub>1</sub> more efficiently. However autoclaving affected bacteria structure severely because of the heat sensible compound such as peptidoglycan and polysaccharides in bacteria cell wall. These changes in cell wall of Lactobacillus by autoclaving it could affect the binding sites of mutagens such as AFB<sub>1</sub> in surface of bacteria. The efficient removal of AFB<sub>1</sub> by *Lb. plantarum* in logarithmic growth phase is important while the growth inhibition ability of this probiotic strain against most fungi strains had been proved (Lavermicocca *et al.*, 2000). Ability of growth inhibition of toxigenic strain of fungi and remove of their produced toxins from media can be a biological method to control foods contaminations. Also in this study we tried to use a different concentration of AFB<sub>1</sub> (0.5 ppm) from what El-Nezami and others (5 ppm) were used. We tried to examine almost the real concentration of AFB<sub>1</sub> which will be contaminated the foods and agricultural crops.

In this study incubation time was the condition which we tried to optimise. El-Nezami *et al.* (1998a) reported that viable *Lb. plantarum* (ATCC 8014) removed about 29.9% of AFB<sub>1</sub> from solution (5 ppm) after 4 h in 37°C. This study revealed that by using *Lactobacillus* in growth phase and increasing of incubation time better results will be obtained since the bacterial surface will change during growth.

Morotomi has been reported, both reversible and irreversible binding of mutagens to lactic acid bacteria (Morotomi *et al.*, 1986; Orrhage *et al.*, 1994). *Lb. plantarum* (PTCC 1058) showed weakly reversible binding of AFB<sub>1</sub> when washed with PBS (Table 1). There wasn't considerable variation in the percentage of AFB<sub>1</sub> bound both initially and after up to three washes. *Lb. plantarum* (PTCC 1058), was effective in initially binding and also retaining AFB<sub>1</sub>, suggesting that the complexes formed with this strain were the most stable. However AFB<sub>1</sub> reduction by autoclaved bacteria wasn't stable, while by increasing incubation time from 24 to 90 h the percentage of AFB<sub>1</sub> reduction became lower (from 31 to 15%).

## CONCLUSIONS

The less reversibility of binding was demonstrated by the effect of bacterial washing. This suggests that AFB<sub>1</sub> in compare with other *Lactobacillus* have been tested in previous studies is bound to the bacteria more effective. Results from treating AFB<sub>1</sub> solutions in growth phase revealed that the ability of *Lb. plantarum* (PTCC 1058) in AFB<sub>1</sub> reduction became better, but the reversibility of binding by autoclaved bacteria was revealed.

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