

BIOCONTROL OF *ASPERGILLUS FLAVUS* AND AFLATOXIN B1 PRODUCTION IN CORN

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ABSTRACT

The potent mycotoxin aflatoxin B1 is a secondary metabolite of *Aspergillus* fungi that grow, on a variety of food and feed commodities at any stage during growth, harvest, storage and transportation. The occurrence of aflatoxin contamination is global, with severe problems especially prevalent in developing countries. In present study, corn samples were contaminated with aflatoxin B1 in the concentration of 240 µg/kg. Four trials were inoculated by *Lactobacillus plantarum* (PTCC 1058). Three control assays were analysed in the same conditions. All the assays were kneaded and incubated for 4-7 days at 37°C. Aflatoxin B1 was determined after extraction by HPLC. Results showed a drastic removal of the mycotoxin with a reduction of 77 % for Aflatoxin B1 by *Lactobacillus plantarum*. In the inoculated corns, spore germination of *A. flavus* was totally inhibited. Results in inoculated spikes showed a high percentage of reduction of aflatoxin after incubation by *Lb. plantarum*. Gram staining of a sample from inoculated corns and microscopic observation demonstrated that the growth of *A. flavus* spores was totally inhibited by *Lb. plantarum*. Fungal spores were surrounded by *Lactobacillus plantarum* and spores were degraded.

Key words: Biocontrol, Aflatoxin B1, *Lactobacillus plantarum*, corn, *Aspergillus flavus*

INTRODUCTION

Aspergillus flavus and *Aspergillus parasiticus* are the most important aflatoxin-producing filamentous fungi. They can occur in several plant products, like spices, cereals and oily seeds (Jelinek 1998, Pittet, 1998 and Lewellyn, 1992). Aflatoxins (AF) are secondary metabolites with a high carcinogenic potential, especially in liver tissue. In addition the aflatoxins possess an acute toxicity at higher concentrations. The high health risk caused by aflatoxins leads to strict concentration limits in different countries. The USA Food and Drug Administration established 20 ppb as the minimum acceptable level of aflatoxin in all foods other than milk. The European Union has banned the import of peanut with >2 ppb of AFB1 content and with >4 ppb of total aflatoxins in nuts prepared for human consumption. To export tree nuts to the European market, aflatoxin levels should be <3 ppb (Schatzki, 2001).

Several works were carried out on the removal and/or the detoxification of aflatoxin (D'souza, 2000) and some microorganisms were reported to be capable of degrading AFB1 such as *Flavobacterium aurantiacum* by enzymatic way (Ciegler, 1966). Some strains of lactic acid bacteria (LAB) were found to be active in removing AFB1 from contaminated media by the contact method without further incubation (El-Nezami, 1998). During the last century it was realized that the lactic acid bacteria are responsible for the fermentation, and through that the bio-preservative effect, utilized in many food and feed processes. Bio-preservation refers to extended shelf life and enhanced safety of foods using the natural or added micro-flora and their antimicrobial products. Besides lactic acid, several other antimicrobial compounds are produced during growth of LAB (Lindgren, 1990). There are many reports on the production of antibacterial compounds by these bacteria (Lindgren, 1990, 1996), but reports on inhibition of

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yeasts and molds are comparatively few. Magnusson and Schnürer (2001) described production of a proteinaceous antifungal compound by a *Lactobacillus coryniformis* strain, while Lavermicocca *et al.*, (2000) reported isolation of the antifungal compounds phenyl-lactic acid and 4-hydroxyphenyllactic acid from *Lactobacillus plantarum*. *Lb. plantarum* can produce fungus-inhibitory low-molecular-weight substances, such as benzoic acid, methyl-hydration, mevalonolactone, and cyclic dipeptide cyclo (glycyl-L-leucyl) (Niku-Paavola, 1999), while a fungistatic bacteriocin-like substance, pentocin TV35b, was isolated from a *Lb. pentosus* strain (Okkers, 1999). Research dealing with fungal inhibition by lactic acid bacteria and the compounds produced by these bacteria is still novel.

The objectives of this study were to find native lactic acid bacteria strain (*Lactobacillus plantarum* (PTCC 1058)) that efficiently binds aflatoxin B₁, has the ability to collect and reduction of AFB₁ from corn.

MATERIALS AND METHODS

Bacteria and fungi strains and growth conditions

The aflatoxinogenic strain *A. flavus* MAS 915 collected from the mycotoxins laboratory of plant pest's disease research institute in Iran has been used. This strain is able to produce all kinds of aflatoxins especially aflatoxin B₁ (AFB₁). The strain was generally grown in malt extract medium (Hi-Media, India) at 30°C for 72 h. *Lactobacillus plantarum* (PTCC 1058) was studied for biocontrol of aflatoxin B₁ was originally obtained from Persian Type Culture Collection (PTCC) of Iranian Research Organization for Science and Technology in Tehran-Iran. Bacterial strain was cultivated in the Man de Rogosa sharp broth (MRS, Hi-media, India) for 24 h at 37°C in 5% CO₂ followed according to El-Nezami, *et al.*, (1998). Bacteria concentration in culture flask was performed using spectrophotometer assay in 600 nm (Unicam 5625 UV/VIS) to obtain concentration around 9×10^9 cfu/mL. Culture broth of MRS was washed with PBS (pH 7.3, 0.01 M) before inoculation to corn samples.

Standard aflatoxin preparation

AFB₁ powder was suspended in methanol in order

to make 10 µg/mL stock solutions. This concentration of the stocks was calculated by recording the UV spectrum (Shimadzu UV spectrophotometer, 160A). The solution was stored at -20°C in the dark.

Contamination of corn sample by aflatoxinB1 (spike)

10 g of corn samples were contaminated with the stock aflatoxin solutions to obtain a final concentration of AFB₁ around 240 µg/kg and the solvents were allowed to evaporate overnight. *Lactobacillus plantarum* suspensions (9×10^9 cfu/mL) was added and incubated at 37°C for 4 days. Three control assays were analysed in the same conditions (corn contaminated and not inoculated, corn not contaminated but inoculated, corn not contaminated and inoculated).

Corn contamination by Aspergillus flavus and bio-detoxification assays

10 g of corn samples were prepared in Petri dishes and autoclaved two times in intervals of 24h and then they were inoculated with 10^6 freshly prepared spores/g, placed in a jar and a layer of water to increase the moisture content of the whole system and incubated at 30°C for 24 h. *Lb. plantarum* suspension in PBS (9×10^9 cfu/mL) was added to each corn samples and all were incubated for 7 days at 37 °C. All samples were in duplicate and blank for corn (corn not inoculated by spore of fungi and *Lactobacilli* and corn inoculated only by *Lactobacilli*).

Aflatoxin determination by HPLC assay

AFB₁ was extracted and purified according to the method was described in the AOAC, 1999. Samples were added to 60 mL methanol: water (8:2) solution in Erlenmeyer flask and shaken for 30 min in room temperature. The extract was filtered through Whatman filter paper. 10 mL of filtered solution was diluted by 70 mL of PBS and filtered again through a fibreglass filter to separate the pigments of corn. The immuno-affinity columns (Aflatest, R-biopharm, France) were used for clean up procedure prior to High Performance Liquid Chromatography (HPLC). The HPLC system consisted of a pump solvent delivery system. Aflatoxins were resolved using Prevail C-18 HPLC column and UV detected at 365 nm

(Manda, 2004). The mobile phase was water: acetonitrile: methanol at a ratio of 58:21:21. The flow rate was 1.25 mL/min.

RESULTS

Lactobacillus plantarum (PTCC 1058) exhibited high reduction of AFB1 from corn contaminated (77%) after three days by HPLC analyses. Growth of *Aspergillus flavus* was totally inhibited in inoculated corns by *Lb. plantarum*, while after 2 days, in blank sample the maximum growth of *A. flavus* was observed (Fig. 1). During 7 days

incubation, there was no fungal growth observed in bacterial inoculated samples. The toxinogenic ability of *A. flavus* was ensured by HPLC chromatogram after extraction from not bacterial inoculated sample after 7 days incubation (Fig. 2). Gram staining of a sample from inoculated corns and microscopic observation demonstrated that the growth of *A. flavus* spores was totally inhibited by *Lb. plantarum*. Fungal spores were surrounded by *Lactobacillus plantarum* and spores were degraded (Fig. 3).

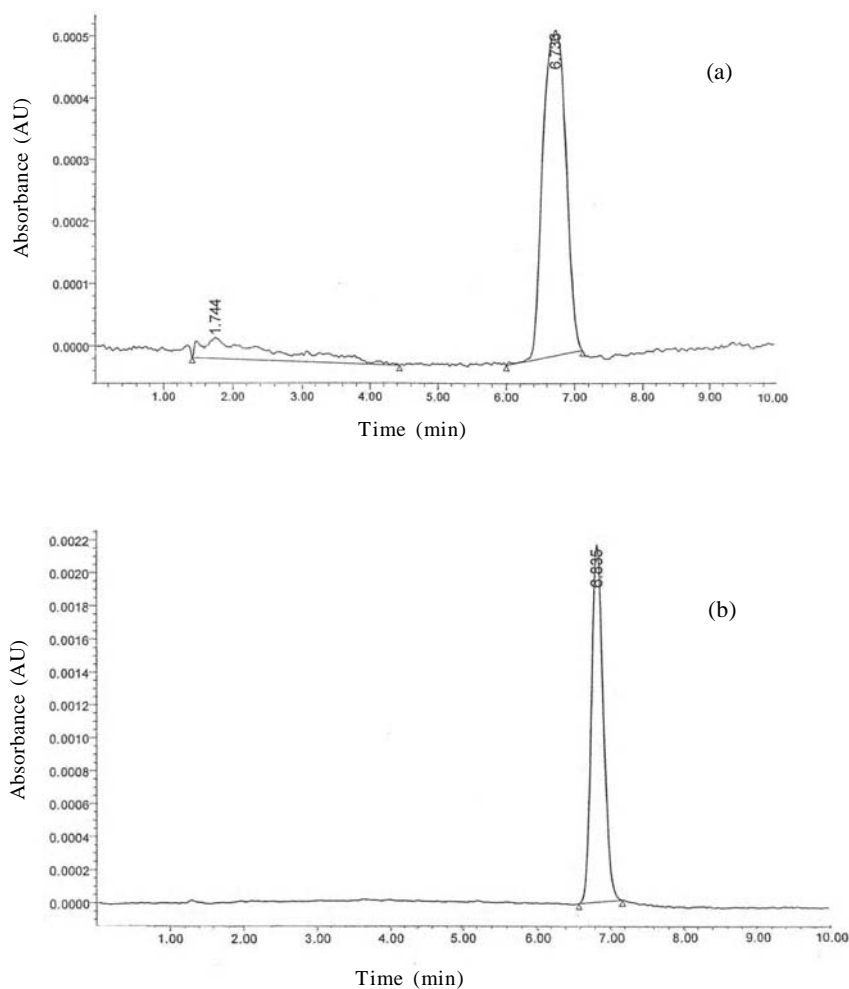


Fig. 1: HPLC profiles of extracts from inoculated spike corns

(a) blank, (b) sample. Growth of *Aspergillus flavus* was totally inhibited in inoculated corns by *Lb. plantarum*, while after 2 days in blank sample the growth of *A. flavus* was observed

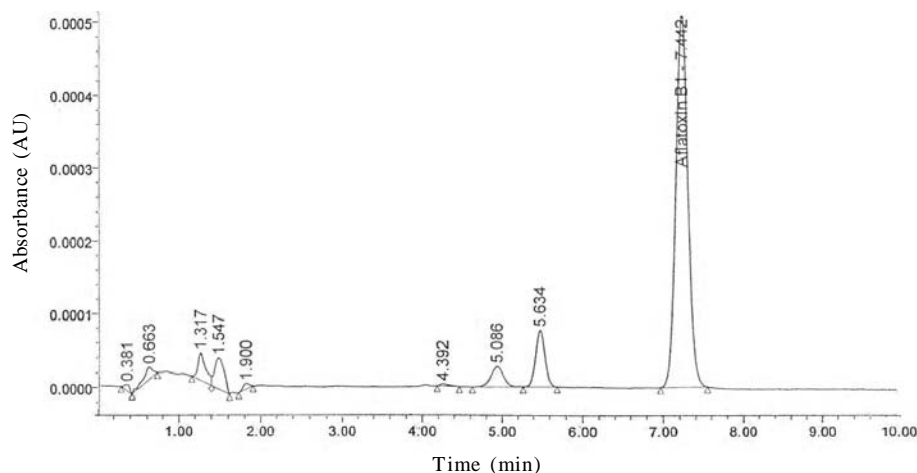


Fig. 2: HPLC profile of corn contaminated with *Aspergillus flavus* spores extracts. The toxinogenic ability of *A. flavus* was ensured by HPLC chromatogram after extraction from not bacterial inoculated sample after 7 days incubation

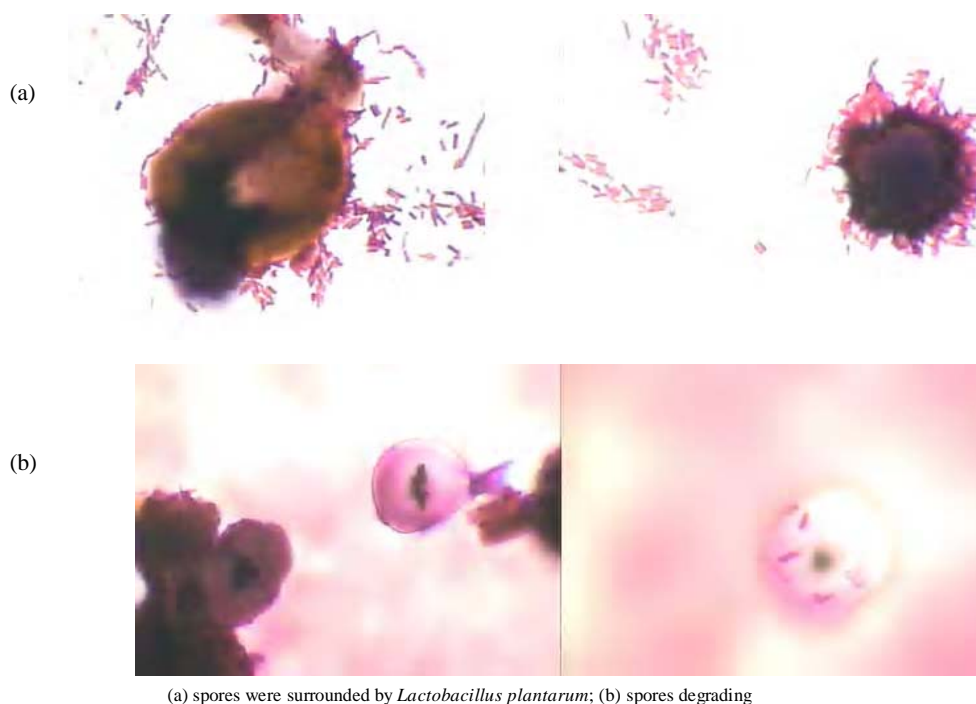


Fig. 3: Gram staining of *A. flavus* spores samples inoculated with *Lactobacillus plantarum*

DISCUSSION

This study demonstrated the ability of *Lactobacillus plantarum* to reduce amounts of aflatoxin B1 in contaminated corns. The amounts of decrease of AFB1 are more important in all assays used. Research using isolates of lactic acid

bacteria to reduce aflatoxins in vitro is essential to determine and identify strains that have the ability to reduce aflatoxins. Furthermore it is important to study the effect of this isolates in silages on aflatoxin reduction. Several strategies for the elimination or inactivation of mycotoxins have been

reported in the literature (Galvano, 2001). Nevertheless, only few of them have been accepted for practical use (ammonia treatment) and no one is entirely effective. Some specialists are of the opinion that the best approach for decontamination of mycotoxins should be degradation by selected microorganisms (Bata, 1999).

LAB with antifungal activities could have potential as bio-preservatives, preventing growth of spoilage molds and yeasts in food and feed systems. In this study we reported the antifungal ability of *Lb. plantarum* (PTCC 1058). In the inoculated corns, spore germination of *A. flavus* was totally inhibited. Results in inoculated spikes showed a high percentage of reduction of aflatoxins after incubation by *Lb. plantarum*. This suggested that lactic acid bacteria may be involved in reduction process. This is in agreement with other researches carried out on the interactions between lactic acid bacteria and aflatoxins (El-Nezami, 1998, Peltonen, 2001). Lactic acid bacteria are shown to be important constituents of silage fermentations and they were responsible of removal processes of aflatoxins in liquid solutions and foods in several studies. Megalla and Mohran (1984) have demonstrated the ability of *Lc. lactis* ATCC 11454 to transform AFB1 in fermented milk into aflatoxicol and aflatoxin B2a. Rasic *et al.*, (1991) have observed a decrease of amounts of AFB1 in yoghurt and acidified milk. AFB1 added to milk before fermentation at concentrations of 600, 1000 and 1400 µg/kg was reduced in yoghurt (pH=4) by 97, 91 and 90%, respectively. El-Nezami *et al.*, (1998) reported that strains of *Lb. rhamnosus* GG and *L. rhamnosus* Lc 705 reduce amount of AFB1 (80%) by a rapid process (24 h) from liquid media. More recently, investigations have reported that dairy strains of lactic acid bacteria and *Bifidobacteria* spp have the ability to bind aflatoxin B1 from contaminated solution; binding was reversible and AFB1 was released by repeated aqueous washes (Haskard, 2001, Peltonen, 2001). Even though the mechanism of AFB1 removal by Lactic acid bacteria is still unknown, it has been suggested that aflatoxins molecules are bound to the bacterial cell wall components of bacteria. Haskard *et al.*, (2001) suggested that AFB1 is bound to the bacteria by weak non-covalent

interactions, such as associating with hydrophobic pockets on the bacterial surface. Peltonen *et al.*, (2001) showed the ability of both strains of lactic acid bacteria and strains of *Bifidobacteria* sp to remove the AFB1 from contaminated solution, the binding process was reversible and AFB1 was released by repeated aqueous washes. Recently, Khanafari, *et al.*, (2007) reported that for *Lb. plantarum* in aqueous AFB1 standard solutions, reduction of AFB1 was more stable as other tested strains of LAB in other studies, while after 3 times of washes about 8.7% of removed AFB1 were released back to washing solutions. Ability of *Lb. plantarum*, toxinogenic fungi spores' germination inhibitor and AFB1 removal, introduced it as an effective bio-preservative in silages. Further evaluation of LAB for antifungal properties could lead to useful bio-preservation systems, preventing fungal spoilage and mycotoxin formation in both food and animal feed.

REFERENCES

- Bata, A., Lastzity, R., (1999). Detoxification of mycotoxin contaminated food and feed by microorganisms. Trends. Food. Sci. Technol., **10**: 223-8.
- Ciegler, A., Lillehoj, E. B., Perterson, R. E., Hall, H. H., (1966). Microbial detoxification of aflatoxin. App. Microbiol., **14**: 934 - 939.
- D'souza, D. H., Brackett, R. E., (2000). The influence of divalent cations and chelators on aflatoxin B1 degradation by *Flavobacterium aurantiacum*. Food. Prot., **63** (1): 102-105.
- El-Nezami, H. S., Kankaanp, P., Salminen, S. J., Ahokas, J. T., (1998). Ability of dairy strains of acid lactic bacteria to bind food carcinogens. Food. Chem. Toxicol., **36**: 321-326.
- Galvano, F., Piva, A., Ritlen, A., Galvano, G., (2001). Dietary strategies to counteract the effects of mycotoxins, A review. J. Food. Prot., **64**: 120-31.
- Haskard, C. A., El-Nezami, H. S., Kankaanää, P. E., Salminen, S., and Ahokas, J. T., (2001). Surface binding of aflatoxin B1 by lactic acid bacteria. Appl. Environ. Microbiol., **67** (7): 3086-3091.
- Jelinek, C. F., Pohland, A. E., and Wood, G. E., (1989). Worldwide occurrence of mycotoxins in foods and feeds— an update. J. Assoc. Anal. Chem. **72**: 223-230.
- Khanafari, A., Soudi, H., Miraboulfathi, M., Karamei Osboo, R., (2007). An In vitro Investigation of Aflatoxin B₁ Biological Control by *Lactobacillus plantarum*, Pakistan Journal of Biological Sciences, in press.
- Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., and Gobetti, M., (2000). Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl. Environ. Microbiol. **66**: 4084-4090.

- Lewellyn, G. C., Mooney, R. L., Cheattle, T. F., and Flannigan, B., (1992). Mycotoxin contamination of spices an update. *Int. Biodeterior. Biodegrad.* **29**: 111-121.
- Lindgren, S.E., and Dobrogosz, W.J., (1990). Antagonistic activities of lactic acid bacteria in food and feed fermentations. *FEMS Microbiology Reviews.*, **87**: 149-164.
- Magnusson, J., and Schnürer, J., (2001). *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Appl. Environ. Microbiol.* **67**: 1-5.
- Manda, A., Naidu, B. P., Nageswara, R., Rachaputi, C., Graeme, W., and Shu, F., (2004). Aflatoxins and their relationship with sugars in peanut (*Arachis hypogaea*). New directions for a diverse planet: Proceedings of the 4th International Crop Science Congress; Brisbane, Australia, 26 Sep- 1 Oct 2004/ ISBN 1 920842 20 9/ www.cropscience.org.
- Megalla, S. E. and Mohran, M. A., (1984). Fate of Aflatoxin B1 in fermented dairy products. *Mycopathologia*, **88**: 27-29.
- Niku-Paavola, M. L., Laitila, A., Mattila-Sandholm, T., and Haikara, A., (1999). New types of antimicrobial compounds produced by *Lactobacillus plantarum*. *J. Appl. Microbiol.* **86**: 29-35.
- Okkers, D. J., Dicks, L. M., Silvester, M., Joubert, J. J., and Odendaal, H. J., (1999). Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with a fungistatic effect on *Candida albicans*. *J. Appl. Microbiol.* **87**: 726-734.
- Peltonen, K., El-Nezami, H., Haskard, C., Ahokas, J., and Salminen, S., (2001). Aflatoxin B1 binding by dairy strains of lactic acid bacteria and bifidobacteria. *Dairy Sci.*, **84** (10): 2152-2156.
- Pittet, A., (1998). Natural occurrence of mycotoxins in foods and feeds—an updated review. *Rev. Vet. Med.*, **149**: 479-492.
- Rasic, J. L., Skrinjar, M., and Markov, S., (1991) Decrease of aflatoxin B1 in Yogurt and acidified milks. *Mycopathologia*, **113** (2): 117-119.
- Schatzki, T. F., (2001) Dependence of aflatoxin in almonds on the type and amount of insect damage. *J. Agri. Food. Chem.*, **49**: 4513 – 4519.
- Stiles, M. E., (1996). Biopreservation by lactic acid bacteria. *Antonie Leeuwenhoek*, **70**: 331-345.