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Assessing the ability of *Lactobacillus rhamnosus* GG to bind aflatoxin B_1 from contaminated cottonseed

Parya Rahnama Vosough[1], Ali Mohamadi Sani[2]*, Masoomeh Mehraban Sangatash[3], Reza Karazhyan[3]

[1]Food Science and Technology Department, Islamic Azad University, Quchan Branch, (IRAN)

[2]Food Science and Technology Department, Quchan Branch, Islamic Azad University, Quchan, (IRAN)

[3]Food Science and Technology Institute, ACECR Mashhad Branch, (IRAN)

E-mail: rahnama_parya@yahoo.com; mohamadisani @yahoo.com; mmehrabans@yahoo.com; reza_karazhyan2002@yahoo.com

ABSTRACT

Biological decontamination of mycotoxins using microorganisms is one of the well known strategies for the management of mycotoxins in foods and feeds. In this study, the interaction of aflatoxin B_1 (AFB_1) in cottonseed with *Lactobacillus rhamnosus* strain *GG was* investigated for the first time. AFB_1 at concentrations (5, 10 and 20 µg/l) was added to the cottonseed meal in buffer phosphate solution and then bacterial culture (10^9 CFU/ml) in MRS broth medium was added to the solution and incubated at 25°C for 4, 12 and 24 hrs. The aflatoxin binding capacity of the strain was quantified by the amount of unbound AFB_1 using ELISA technique. Results showed the binding capacity of viable, heat killed and acid killed bacteria respectively 44, 47 and 49%. Removal of AFB_1 by this strain was a slow process with approximately 41% AFB_1 removal at both 12 and also 24 hrs. The primary concentration of AFB1 did not influence the efficacy of detoxification ($p>0.05$). These findings further support the ability of specific strains of lactic acid bacteria to bind feed contaminants.

KEYWORDS

Aflatoxin B_1;
Lactobacillus rhamnosus;
ELISA;
Cottonseed.

INTRODUCTION

Mould growth in agricultural products may cause an important hazard to human health by the formation of toxic metabolites called "mycotoxin". Aflatoxins (AFs) belong to the group of mycotoxins[24]. AFs are a group of highly toxic secondary metabolite products of *Aspergillus flavus*, *A.parasiticus* and *A.nomius* and have carcinogenic and teratogenic effects to livestock and human[29]. *A.flavus* and *A.parasiticus* are ubiquitous fungi, showing particular affinity for oily seeds as a growth source. Main sources of aflatoxins in feeds are peanut, maize and cottonseed meals[26]. The four major aflatoxins are B_1, B_2, G_1 and G_2 based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin layer chromatography (TLC)[22]. Chronic exposure to low levels of AFB_1; the most potent aflatoxin, poses a serious health and economic hazard[19]. Production of mycotoxins by toxigenic mold species contaminating food and feed depends on

several environmental factors, for example temperature, humidity and other storage conditions[27]. Contamination of agricultural crops with AFs is a worldwide problem not limited to developing countries, where both climatic and technological conditions stimulate aflatoxin formation[2]. When animals eat foodstuffs containing AFB_1, these toxins will be metabolize and excrete as aflatoxin M_1 (AFM_1) in milk. There is a general consensus that approximately 1-3% of the AFB_1 initially present in the animal feedstuff appears as AFM_1 in milk[1,5]. AFM_1 is cytotoxic, as demonstrated in human hepatocytes in vitro. This mycotoxin can also cause DNA damage, gene mutation, chromosomal anomalies and cell transformation in mammalians cells in vitro. However, AFM_1 is less mutagenic and genotoxic than AFB_1[9,26]. Since milk has the greatest demonstrated potential for introducing AFs residues from foods of animal origin into the human diet and is also the main nutrient for infants and children, the occurrence of AFM_1 in milk and dairy products is a concern[20]. Various physical and chemical methods have been used to detoxify AFs from feed materials. The use of many of the available physical and chemical methods for detoxification of agricultural products contaminated with mycotoxins is restricted due to problems concerning safety issues, possible losses in nutritional quality of treated commodities, coupled with limited efficacy and cost implications. This has led to search for alternative strategies such as biological agents[6,7,15,18,29]. Bacteria like *lactobacillus* strains have been tested on their ability to inactivate AFs[10]. The aim of this study was to investigate the possibility of removing AFB_1 by *Lb. rhamnosus GG* from contaminated cottonseed meal.

MATERIAL AND METHOD

Bacterial strain, culture conditions and estimation of bacterial concentration

Lactobacillus rhamnosus strain *GG* was used for AFB_1 detoxification. The strain was obtained in lyophilized form from Iranian Research Organization for Science and Technology (IROST), Tehran (Iran). *Lb. rhamnosus* was activated and propogated in MRS broth (Merck, Germany) at 37°C for 24 hrs. After incubation, cells were collected by centrifugation (3400g, 10 min, 4°C) and washed twice with phosphate buffered saline (PBS, pH 7.2). To obtain suspensions with concentrations of 10^9 CFU/ml, Mc-Farland solution was used[13]. Estimation of bacterial concentrations was performed using a spectrophotometer and adjusting the optical density at 600 nm[10,23]. Bacterial suspensions (10^9 CFU/ml) were either used as viable, heat treated (autoclaved at 121ÚC in PBS for 15 min) and acid treated (incubated at 37°C in 10 ml 2 M HCl for 1 hr)[11].

Preparation of AFB_1 working solution

Solid AFB_1 (sigma) was suspended in benzene/acetonitrile (93:7 v/v) to obtain a concentration of approximately 100 µg/ml. To prepare an aqueous solution, benzene/ acetonitrile was evaporated by heating in water bath (80°C for 10 min) and AFB_1 was suspended again in methanol to make a final concentration of 1 µg/ml.

Contamination of cottonseed Samples by AFB_1

The uncontaminated cottonseed sample was milled and 5 grams samples suspended in 20 ml PBS. The samples were contaminated with 5, 10 and 20 µg/l AFB_1 and 10 ml of bacterial suspension (10^9 CFU/ml) was added to them and incubation was done at 25°C for 4, 12 and 24 hrs. Finally, samples were centrifuged (7500g, 10 min, 25°C) and supernatant was quantified for AFB1 detection by ELISA technique. Control assays (cottonseed contaminated by AFB1 but not inoculated by bacterial suspension) were analyzed in the same conditions[16].

Quantification of AFB_1 by ELISA technique

According to Europroxima AFB_1 (Art No.5121) test kit manual, 50 µl aflatoxin standard solutions and 50 µl samples were added into wells in duplicate. Then, 25 µl of the diluted conjugate (Aflatoxin-HRP) and 25 µl of the antibody solution were added to each wells, except wells A_1 and A_2. The plate was incubated for 1 hour at 37°C. The liquid was then removed completely from the wells, and each well was washed with rinsing buffer. The washing procedure was repeated for three times in ELISA washer (ELX 50, Bio-Tek Inst.). After the washing step, 100 µl of substrate solution was added to each well and incubated for 30 min. at room temperature in the dark. The reaction was stopped by adding 100 µl of the stop solution to each well and the absor-

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bance was measured at 450 nm in ELISA plate reader (ELX 808, Bio-Tek Inst.).

Statistical analysis

Data were analysed as a completely randomized factorial design. The mean analysis was done for determining binding amount of AFB_1 in cottonseed in SPSS 16. Significant differences in the mean values were reported at $p \leq 0.05$.

RESULT AND DISCUSSION

Several potentially feasible strategies for the reduction on inactivation of aflatoxins have been reported in the scientific literature. Some methods are clearly more effective and practical than others; most reduce the levels of parent aflatoxins or modify the toxicity associated with these poisons to some degree. Aflatoxin may be degraded by physical, chemical or biological methods. AFB_1 was selected because of its wide occurrence and detrimental effects on human and animal health even in minor quantities. During the last two decades, several studies have suggested that lactic acid bacteria and fermented dairy products possess anti-carcinogenic activity. Lactic acid bacteria are noted for their ability to bind mutagens[8,14]. Our attention has been focused on the binding ability of *Lactobacillus rhamnosus* strain GG to AFB_1 in an artificially contaminated feed. The ability of this strain to bind AFB_1 in PBS as viable and non-viable preparations and at different AFB_1 concentrations and different incubation times are summarized in TABLE 1, 2 and 3.

TABLE 1 : Percentage AFB_1 bound on exposure to viable bacteria in different concentration of AFB_1 at different incubation time.

	Viable bacteria			
	0h[a]	4h	12h	24h
5 µg/l[b]	29.8±0.56[c]	64±2.12	71.4±1.7	72.8±0.56
10 µg/l	29.8±1.27	64±1.06	72±0.71	71.3±0.56
20 µg/l	29.8±0.92	64±0.71	71.15±0.81	70.95±0.32

Incubation time; b. Concentrations of AFB_1; c. Results are the mean ± SD for duplicate samples.

Effect of heat and acid treatments on AFB_1 binding ability

Results in Figure 1 show significant differences in remotion of the toxin by viable and non-viable (acid- and heat-treated) cells. Acid-treated bacteria removed the highest amount of AFB_1 ($p<0.05$).

TABLE 2 : Percentage AFB_1 bound on exposure to viable bacteria in different concentration of AFB_1 at different incubation time.

	Heat treated bacteria			
	0h[a]	4h	12h	24h
5 µg/l[b]	33.7±1.91[c]	72±1.41	74.4±1.7	75±1.41
10 µg/l	33.7±0.92	72±1.06	74.4±0.99	73.8±1.27
20 µg/l	33.7±1.55	72±0.35	73.6±0.78	73.45±1.02

Incubation time; b. Concentrations of AFB_1; c. Results are the mean ± SD for duplicate samples.

TABLE 3 : Percentage AFB_1 bound on exposure to viable bacteria in different concentration of AFB_1 at different incubation time.

	Acid treated bacteria			
	0h[a]	4h	12h	24h
5 µg/l[b]	37.6±1.89[c]	74.4±0.99	71±0.71	76.6±1.84
10 µg/l	37.6±1.48	74.4±0.99	76±1.06	75.4±1.34
20 µg/l	37.6±0.42	74.4±1.17	75.25±1.24	73.5±0.35

Incubation time; b. Concentrations of AFB_1; c. Results are the mean ± SD for duplicate samples.

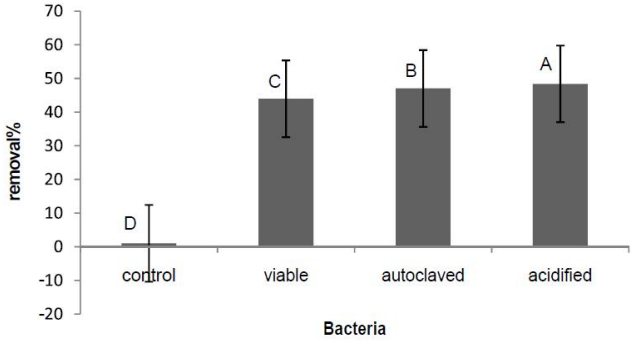

Figure 1 : Effect of bacterial heat and acid treatment on the removal of AFB_1.

Heat treated LAB have previously been shown to effectively bind aflatoxins[4,28]. Peltonen et al.[23] showed that heat and acid treatments markedly increased the bacterial AFB_1 binding ability. Haskard *et al.*[11] revealed that heat and acid treatments also significantly enhanced the ability of *Lb. rhamnosus* strain GG (A53103) and *Lb. rhamnosus* strain LC-705 (DSM7061) to remove AFB_1 from contaminated defined medium, with acid treatment being more effective than heat treatment in most cases. El-Nezami *et al.*[4] reported that the binding ability increased by acid treatment. In another study

El-Nezami et al.[4] indicated that heat-treated dairy strains of *lactic acid bacteria* has the same ability to remove AFB_1 as viable bacteria.

Effect of AFB_1 concentration on the rate of detoxification

Effect of different AFB_1 concentrations on toxin removal by viable and non-viable bacteria is shown in Figure 2. The percentage of AFB_1 removed in different concentration was not significantly different ($p<0.05$).

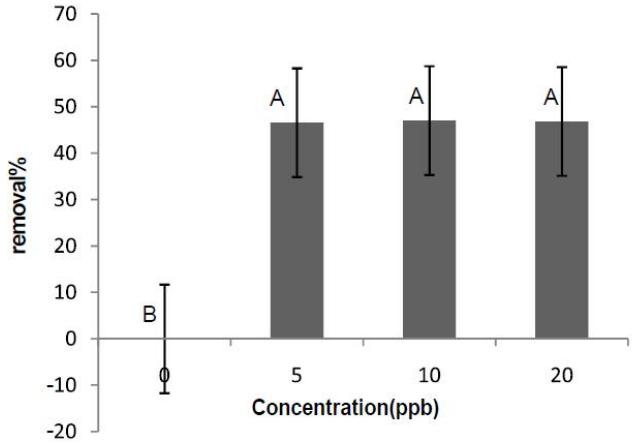

Figure 2 : Effect of AFB_1 concentration on the removal of AFB_1.

According to El-Nezami et al.[4] the amount of AFB_1 removal increased with increasing concentration of AFB_1 but the percentage removed was not significantly different. It contrasted with findings by Line and Brackett where the percentage removal of AFB_1 decreased as toxin levels increased. Also Pizzolitto et al.[25] showed that *Lb. rhamnosus1, Lb. acidophilus24* and *Lb. casei* subsp. *rhamnosus* were the best binders at 50, 100 and 500 $ng.ml^{-1}AFB_1$, respectively. Lee et al.[17] refer to AFB_1 binding as a process of very high-affinity, linear relation with the toxin concentration used, and therefore, the amount of AFB_1 bound should be limitless; in other words they concluded that the bacterial surface does not have a defined number of binding sites.

Also the results showed that the initial AFB_1 concentration had no significant effect ($p<0.05$) on the rate of detoxification by viable and non-viable bacteria (Figure 3). Acid-treated bacteria bound AFB_1 significantly compared with heat-treated and viable bacteria. However Haskard et al.[17] showed that the relative amounts of AFB_1 removed by viable and nonviable bacteria depended on initial AFB_1 concentration.

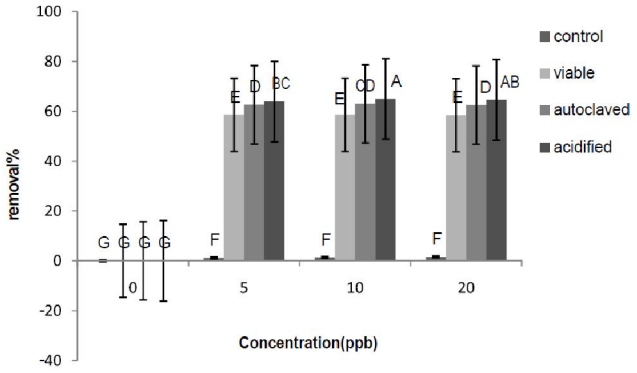

Figure 3 : Interaction effect of bacterial heat and acid treatment in different AFB_1 concentration on the removal of AFB_1.

Effect of incubation time on the reduction of AFB_1

According to Figure 4 by varying the incubation time from 0 hr to 12 hrs, significant difference ($p<0.05$) in the amount of AFB_1 removed was observed. The difference in the binding ability of bacteria was not significant between 12 and 24 hrs.

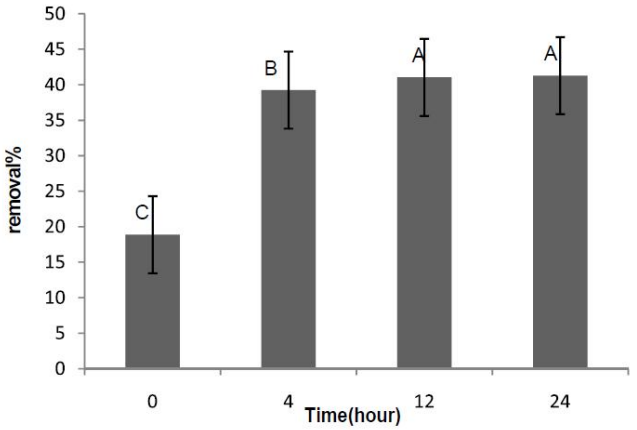

Figure 4 : Effect of incubation time on the removal of AFB_1.

Peltonen et al.[23] reported that the AFB_1 binding of *Lb. amylovorus* CSCC 5160 was increased significantly ($p<0.05$) with extended incubation time from 52.6% (24hrs) to 73.2% (72hrs), whereas the binding ability of *Lb. rhamnosus* strain $Lc^{1/3}$ remained constant after 24 hrs. El-Nezami et al.[23] showed that the removal of AFB_1 was a rapid process with no significant differences observed between different incubation periods.

According to Figure 5 there is no significant difference between removal rate of AFB_1 at 12 and 24 hrs for viable and heat-treated bacteria. In case of low AFB_1 concentration (5µg/l), the AFB_1 binding increased significantly ($p<0.05$) with extended incubation time, but in higher concentrations (10 and 20 µg/l), the binding

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rate increased after 0 hr and remained constant after 12 hrs (Figure 6).

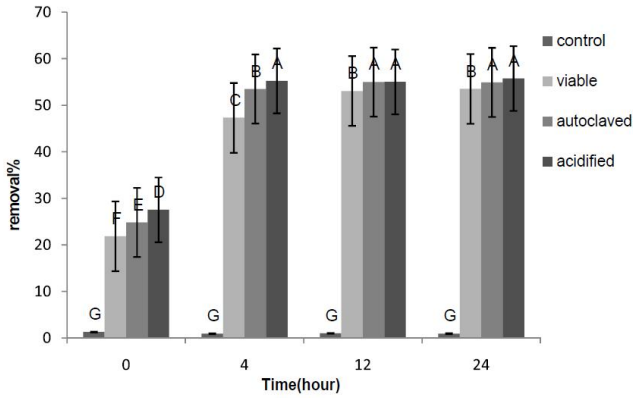

Figure 5 : Interaction effect of bacterial heat and acid treatment in different incubation time on the removal of AFB_1.

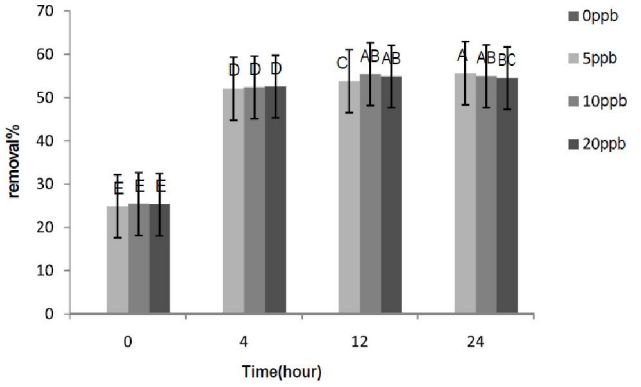

Figure 6 : Interaction effect of AFB_1 concentration in different incubation time on the removal of AFB_1.

CONCLUSION

Animal feed contamination to AFB_1 is unavoidable, therefore a protection against aflatoxicosis is necessary. The present study further supports the observation that specific probiotic *lactic acid bacteria* are able to bind dietary mutagens and carcinogens. It has been clearly shown that bacterial viability is not significant for the removal of AFB_1, as nonviable bacteria significantly reduced more AFB_1 as compared to viable bacteria ($p<0.05$). Furthermore, with increasing incubation time the AFB_1 binding ability increased. An animal feeding trial needs to be conducted with levels of AFB_1 naturally present in feed to evaluate the AFM_1 binding ability in gastrointestinal tract.

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