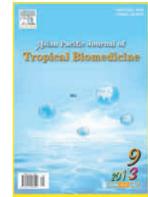




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## The aflatoxin B1 isolating potential of two lactic acid bacteria

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## PEER REVIEW

## Peer reviewer

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## Comments

The present research presented an  
appropriate and useful method in  
finding the new capabilities among  
lactic acid bacteria for isolating and  
binding with the very potent toxin  
belonging to aflatoxin B1.

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## ABSTRACT

**Objective:** To determine lactic acid bacteria's capability to enhance the process of binding and isolating aflatoxin B1 and to utilize such lactic acid bacteria as a food supplement or probiotic products for preventing absorption of aflatoxin B1 in human and animal bodies.

**Methods:** In the present research, the bacteria were isolated from five different sources. For surveying the capability of the bacteria in isolating aflatoxin B1, ELISA method was implemented, and for identifying the resultant strains through 16S rRNA sequencing method, universal primers were applied.

**Results:** Among the strains which were isolated, two strains of *Lactobacillus pentosus* and *Lactobacillus beveris* exhibited the capability of absorbing and isolating aflatoxin B1 by respectively absorbing and discharging 17.4% and 34.7% of the aforementioned toxin existing in the experiment solution.

**Conclusions:** Strains of *Lactobacillus pentosus* and *Lactobacillus beveris* were isolated from human feces and local milk samples, respectively. And both strains has the ability to isolate or bind with aflatoxin B1.

## KEYWORDS

Mycotoxin, Aflatoxin, Lactic acid, Bacteria, ELISA test

## 1. Introduction

Mycotoxins along with other fungal metabolites, similar to antibiotics, are produced by the fungal cells at the final growth stages of the soil–living mycelia (molds). Mycotoxins presented in food can be an important factor for nutritional hazards. In the industrialized nations, more than 10% of the populations may annually suffer from food source–related sicknesses. Aflatoxins are large groups of mycotoxins

that are produced by specific species of molds, including *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, belonging to *Aspergillus* genera and possessing a great global prevalence<sup>[1–3]</sup>.

Aflatoxin B1 (AFB1) has been known as the most potent toxin among various aflatoxins. This compound exerts its toxic effects upon the liver cells and it is responsible for inflicting damages on DNA, mutation, induction of cancer, abortion, birth deformity, suppression of immune system,

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and phytotoxic reactions. Aflatoxins types M1 and M2 are also another pair of toxic metabolites from AFB1. They are also called milk toxins which are secreted through animal milk, such as that of a human, of those who have consumed some quantities of contaminated food laden with AFB1. There is a high degree of possibility for transmittance of these toxins from a mother to her infant. In a numerous studies, a high percentage of contamination of the mothers' milk with these toxins in various regions of the world has been proven<sup>[1,4–7]</sup>.

In addition to all the aforementioned reasons, aflatoxins can also, from the economical aspect, contaminate agricultural exports causing many difficulties for many countries.

There are three known methods of neutralizing aflatoxins present in food: thermal method, chemical method, and microbial method.

In the thermal method, it is possible to destroy and eliminate aflatoxins present in food products through the use of high degrees of temperature, but this method has no application in the food industry for the reason that the high temperature causes changes in food appearance and its taste and eventually ruins its quality. In the chemical method contamination of aflatoxins is eliminated through the utilization of compounds like the derivatives of chlorine (*i.e.*, sodium hypochloride) and the bleaching solutions. This method is inapplicable due to its destructive effect and the toxicity caused by the residues of these chemicals in aflatoxins detoxification. The method is only used in decontamination of the environments polluted with aflatoxins<sup>[1,2]</sup>.

A great chance for reducing the presence of aflatoxins in food products is implemented through the utilization of certain types of nonpathogenic bacteria, such as the group of lactic acid bacteria (LAB). The majority of LAB and the entire genera of *Lactobacillus* are completely safe and nonpathogenic<sup>[8]</sup>.

With isolation of the LAB, especially *Lactobacillus*, which have the capability of binding with aflatoxins and removing them from the suspension, and *Lactobacillus* are the most important human probiotic bacteria and they are completely safe and nonpathogenic. Therefore, it is feasible to use them as probiotics. These types of probiotics, through the way of binding with aflatoxins presented in food, are able to prevent the absorption of toxins in the digestive system, which in turn, results in the prevention of aflatoxins toxication and also the secretion of aflatoxins types M1 and M2 in humans and animals milk. Through the use of strains of LAB, it is possible to perform aflatoxins detoxification without causing any kind of problems associating with the food quality and the presence of materials toxic residues. Moreover, the addition of these substances causes an increase in the health-giving effect of milk and its dairy products, and also in strengthening the intestine's local microbial flora in humans. Finally culturing these bacteria is facile and a great

volume of them can be produced in a short period of time<sup>[9–13]</sup>.

## 2. Materials and methods

### 2.1. Isolating and culturing of the families of LAB

The samples used in isolating the bacteria of the LAB group in this test were food materials' samples, environmental samples, clinical samples, laboratory samples and samples produced from microbial banks.

To isolate LAB from different sources, two specific mediums of MRS (de Man, Rogosa, Sharpe) broth and MRS agar were used and for the reason that these bacteria are mainly anaerobic or they tend to have a much better growth in the anaerobic conditions, culturing was performed anaerobically in these medium. To perform this task, the test tubes containing MRS broth were initially heated so that their dissolved oxygen exited. In the next stage, liquid samples of 1 mL and solid samples of 1 g were inoculated in the medium of MRS broth and the medium surfaces were covered with paraffin to prevent any oxygen from penetrating into the cultures. The incubator temperature of 37 °C and the period of 4 complete days were considered for the incubation. After the growth of the samples in the liquid medium, they were cultured on the MRS agar dishes and after their colonization, through the Gram stain, catalysis, oxidation, potassium hydroxide 3%, motility, indol and nitrogen reduction tests, the obtained strains association or dissociation to the LAB group was specified<sup>[9]</sup>.

### 2.2. Estimating the bacterial density

Bacterial strains count was performed by the spectrophotometer instrument at the wavelength of 600 nm so that an optical density of  $3.5 \times 10^8$  CFU/mL from each bacterial strain was reached.

### 2.3. Furnishing the concentrates of AFB1

For this study, AFB1 which was in the form of a vial containing 1 g of the toxin powder was purchased from Sigma Company. To reach the approximate density of 10 mg/mL, the powder was dissolved in a suspension of benzene and acetonitrile with a ratio of 97:3. Then in order to obtain the required concentrate, 2 µg/mL, 2 µg of the prepared solution of AFB1 was added to 1 mL of the phosphate buffered saline (PBS) with pH 7.2 and the organic solvent present in the solution was eliminated afterwards through the process of water bathing at 80 °C.

### 2.4. AFB1 bacterial binding test

To determine the LAB bacterial strain's capability in

absorbing AFB1 and its removal from the solution, a quantity of 1 mL of PBS solution containing specific bacterial densities was added to 1 mL of PBS containing a density of 2 µg of AFB1 and the obtained solution was subjected to 37 °C in the shaker incubator with moderate rotation for 48 h.

### 2.5. Determining the residual AFB1 through ELISA test

After exposing the PBS solutions which contained bacterial strains of LAB and AFB1, the solutions were centrifuged for 15 min at 10000 r/min. The bacteria released residues. For the following step, quantities of 500 µL from the upper portions of the samples were removed. For purpose of gaining a greater assurance about the removal of all the bacteria, all the solutions were centrifuged under the previous conditions and quantities of 200 µL from the upper portions of the solutions were removed once again.

ELISA's noncompetitive sandwich kit version for AFB1 was purchased from Veratox (Neogen Company, Canada). The minimum detectable density by the ELISA was at 2 mg/mL. A quantity of 100 µg of the solutions from each sample, as aforementioned was tested by ELISA to ascertain the quantities of the residual AFB1. They were compared against the control solution which were free of any bacteria but contained AFB1. The reductions in the quantities of AFB1 present in the test solutions, with respect to the control solution, was an indication of the capability of the bacterial strains in absorbing the toxin and removing it from the test solutions.

### 2.6. Statistical surveys

For surveying the significance of the findings attained from each of the bacterial strains of LAB which displayed the capability for absorbing the toxin through the ELISA test, a total of 30 new samples were cultured. The new samples similarly underwent detoxification process and were tested by the ELISA to determine whether these strains can again absorb AFB1 at an acceptable level from the solutions. On these basis, the percentage of binding AFB1 for each bacteria can be determined. The results may be studied statistically at the 5% level ( $P < 0.05$  or the assurance of 95%). This test was done on the principles of double equations, using SPSS 16 software.

### 2.7. Identifying the attained strains through 16s rRNA sequencing method

In order to specifically identify the bacterial strains, the 16s rRNA sequencing method was employed and for the purpose of conducting this mission, the DNA of the bacterial strains was derived by the following procedure.

A volume of 1 mL of each cultured sample in MRS broth was transferred into micro tubes and they were centrifuged for 5 min at 5000 r/min. The superficial liquid was emptied

out and to the residue a volume of 200 µL of 10 mmol TrisHCl, 0.1 mol NaCl, and 1 mmol EDTA (pH 8) was added and mixed thoroughly. Then, they were maintained at the lab temperature for 10 min. Next, a volume of 200 µL of 3 mol sodium acetate with pH 5.2 was added. After that, each micro tubes was manually mixed 10 times with hand motion and later they were centrifuged for 5 min at 5000 r/min at 4 °C. The superficial liquid was transferred into other tubes and to their contents for each 3 times as much ethanol 100% (v/v) were added and they were kept at -20 °C for 10 min. The samples were later taken out of the freezer and placed at the lab temperature for 5 min. They were centrifuged for 10 min at 13000 r/min at 4 °C. The superficial liquid of the samples were disposed and a volume of 200 µL of 70% ethanol (v/v) was added and after being placed for 5 min at the lab temperature the micro tubes were centrifuged for 10 min at 13000 r/min at 4 °C. The superficial liquid was disposed and the tubes were held upside down to be dried. After this step, a volume of 50 µL of injectable distilled water was added to each tube's residue and they were maintained in the freezer at -20 °C until PCR test<sup>[14]</sup>.

The sequence for the selected general primers of 16s rRNA in this study are: Primer U3 Position 16S rRNA Sequences 509 – 533 include: 5'-AACTCCGTGCCAGCAGCCCGGTAA3' Sequences, Primer U8 Position 16S rRNA Sequences 1517 – 1541 include: 5'-AAGGAGGTGATCCAGCCGCGAGTTC-3'.

The product of the PCR reaction on the transferred agarose gel 1% underwent electrophoresis for 40 min. The gel then was dyed with a solution of ethidium bromide for 10 min and it was photographed under UV light in the gel doct device. After dying process and observing the gel sheets, uniform bands with equal molecular weights (1000 bp) were formed which were an indication for the reproduction of the 16s rRNA gene segment being under the consideration. In order to determine 16s rRNA gene segment sequencing, the samples were sent to Macrogen Company of South Korea where they were sequenced utilizing Automatic DNA Sequencer 3730XL device. Two of the sent samples possessed the 1518 and 1539 base, and the attained sequences were compared with the other sequences within the Gene Bank by using BLAST N program.

## 3. Results

In all, 100 samples taken from 5 sources as previously mentioned were investigated for separation of LAB and they were all cultured. From their cultures, altogether a total of 119 strains of LAB were isolated. After the detoxification test and consequent determination of the residual quantities of AFB1 through the ELISA method, there were two strains among the other isolates which showed the capability of absorbing AFB1 in the tested solution and their identities were identified through the 16s rRNA sequencing method. The two strains were *Lactobacillus pentosus* and *Lactobacillus*

*berevis* which were isolated from human excrement and the local milk samples, respectively. *Lactobacillus pentosus* and *Lactobacillus berevis* strains bound with 17.4% and 34.7% of AFB1 presented in the test suspension equivalent to 1 µg respectively and remove the toxin (Figure 1).

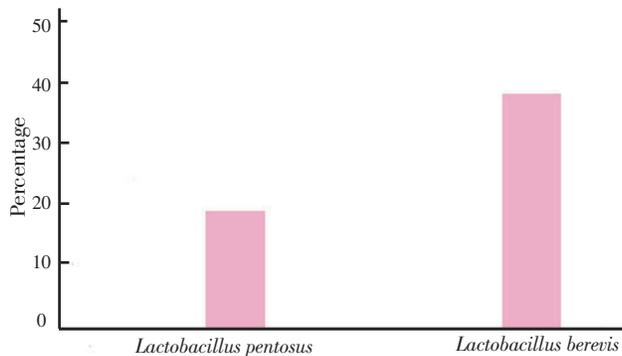


Figure 1. Binding percentage of AFB1 by the isolated *Lactobacillus* strains.

#### 4. Discussion

The total number of AFB1 molecules that can bind to a single bacterium is estimated to be over  $10^7$ . Aside from AFB1, these bacteria also bind with other aflatoxins such as B2, B2a, G1, G2, M1, M2 but not to the extent that they tend to bind with AFB1. Among these aflatoxins, AFB1 type is the most predominant and it is also found most frequently<sup>[9–11]</sup>.

There have been no products obtained from the breakdown of these aflatoxins under HPLC in association with the supernatant or methanol extract of these bacteria. Both strains of these bacteria which are killed by heating and acid are capable of isolating aflatoxins. This finding indicates that the separation of aflatoxins toxins is through the binding process to the surface of the bacteria *i.e.*, teichoic acid component present in the cellular wall, not by the metabolism of the bacteria. Therefore, the living or dead conditions of the bacteria does not play role in isolation the toxin from the test suspension<sup>[15,16]</sup>.

Vast surfaces of the binding sites of the bacteria and various reactions present for AFB1 have been specified. Binding of AFB1 to the surface of LAB is related through the competition among the toxin's access capability towards the binding sites of the bacteria and an antibody for anti-polyclonal AFB1 in ELISA test was specified by indirect competition. Recovery of more than 99% of the binding AFB1 bacteria through the process of extraction by the solvent is also another factor for binding of aflatoxins to the surface of the bacterial<sup>[9]</sup>.

The findings of the present study create an economical and commercial potential for detoxifying the AFB1 contaminated food products through the application of strains of LAB which are normally present in food products. These methods have been studied in both laboratory and inside the food materials so that an industrial process for

separation of aflatoxins from contaminated liquid food products such as milk and oils may be innovated. Similar findings have been reported by other researchers on the capability of LAB in isolating AFB1<sup>[17–23]</sup>.

In conclusion, it is clearly shown in the present study that two strains tested showed a significant ability in reducing levels of AFB1 in liquid media. The ability of these autochthonous *Lactobacillus* strains isolated from Iran to bind AFB1 has the potential to reduce the level of AFB1 in human diet and animal feed.

#### Conflict of interest statement

We declare that we have no conflict of interest.

#### Acknowledgements

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#### Comments

##### Background

Aflatoxins are considered to be a significant problem in the healthfulness of food materials throughout the world. They cause ruination of high quantity of food and create numerous hazards for the health of humans and animals. Among the various types of aflatoxins, type B1 is the most important of all deserving a greater attention, and a great number of aflatoxins are derived from it. A tremendous number of studies have been conducted in order to eradicate or isolate aflatoxins using physical, chemical, and microbial methods. In the present research, the capability of the different strands of lactic acid bacteria in isolating aflatoxin B1 has been studied.

##### Research frontiers

Lactic acid bacteria are mainly found in fermented food materials including dairy and nondairy products and they are utilized as the subjects for scrutinizing in many studies regarding the isolated strains from various food materials. In the present research aside from the food specimens, environmental, clinical and laboratory specimens were used to enable us to isolate certain types of lactic acid bacteria existing in environments other than those belonging to food and to study their capability in extracting and binding with aflatoxin B1.

##### Related reports

The obtained information from the present research

regarding the capability of lactic acid bacteria in binding with aflatoxin B1 concur with the results attained by Fazeli *et al.* (2009) and Haskard *et al.* (2001). The aforementioned researchers have also demonstrated the capability of lactic acid bacteria in binding with aflatoxin B1, of which certain types of lactobacillus possess the capability for isolating aflatoxin B1.

### Innovations and breakthroughs

In the present study, it has been exhibited for the first time that *Lactobacillus pentosus* and *Lactobacillus berevisbacteria* which were isolated from human feces and local milk supply, respectively, had the capability for binding with and extracting aflatoxin B1.

### Applications

The obtained results from the present research possess a great significance with an aim at neutralizing and isolating aflatoxin B1 from food materials for humans and animals through microbial method and it is possible that with exploitation of these two bacteria (*Lactobacillus pentosus* and *Lactobacillus berevis*) and the other lactic acid bacteria whose capability in binding with aflatoxin B1, an applicable method can be innovated for the aforementioned purposes.

### Peer review

The present research presented an appropriate and useful method in finding the new capabilities among lactic acid bacteria for isolating and binding with the very potent toxin belonging to aflatoxin B1. The authors of this research introduced two new bacteria which possess the capability for isolating and binding with aflatoxin B1.

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