

Original Article**Identification of New Aflatoxin B1-Degrading Bacteria from Iran**Fahimeh Sangi¹, Abbas Mohammadi*², Nazar Afzali³, Mansoureh Mirabolfathy⁴

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ABSTRACT

Background: Aflatoxin B1 (AFB1) is a mutagenic and carcinogenic compound mainly produced by the *Aspergillus parasiticus*, *A. flavus*, *A. nomius*, *A. tamari*, and *A. pseudotamarii*. AFB1 biodegradation is the most important strategy for reducing AFB1 in plant tissues. Bacteria can deactivate and biodegrade AFB1 for effective detoxification of contaminated products. The present study investigated the efficiency of AFB1 degradation by soil bacteria from the Southern Khorasan Province in Eastern Iran by thin-layer and high-performance liquid chromatography during 2014–2015.

Methods: DNA was extracted from AFB1-degrading isolates by the cetyltrimethylammonium bromide method and the 16S rRNA gene was amplified with the 27f and 1492r general bacterial primers and the sequences were used to identify the isolates based on their similarity to Gene Bank sequences of known bacterial species.

Results: We isolated five strains from four species of AFB1-degrading bacteria from Birjand plain, including *Bacillus pumilus*, two isolates of *Ochrobactrum pseudogrignonens*, *Pseudomonas aeruginosa*, and *Enterobacter cloace*, which had AFB1-degrading activities of 88%, 78%, 61%, 58%, and 51%, respectively.

Conclusion: We provide the first demonstration of AFB1 degradation by *B. pumilus* in from Iran and the first report identifying *O. pseudogrignonens* and *E. cloace* species as having AFB1-degrading activity.

Keywords: Degrading, Detoxification, *Enterobacter cloace*, *Ochrobactrum pseudogrignonens*, *Pseudomonas aeruginosa*.

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INTRODUCTION

Aflatoxins are bis-furano-isocoumarin compounds produced by some *Aspergillus* species, including *A. parasiticus*, *A. flavus*, *A. nomius*, *A. tamari*, and *A. pseudotamarii* [1, 2]. Among 18 known types of aflatoxins, 14 are produced in nature, with aflatoxin B1 (AFB1) being the most toxic. AFB1 affects DNA, RNA, and protein synthesis and can alter nuclear morphology [3]. *Aspergillus* species can colonize plant tissues under a variety of environmental conditions and produce AFB1 [4], removed from infected tissues by physical and chemical methods [5] to produce resistant plants [6]. AFB1 biodegradation is the most important strategy for reducing AFB1 in plant tissues [4]. Several species of protozoan, fungus, and AFB1-degrading bacterium have been identified to date [4].

The unicellular protozoan *Tetrahymena pyriformis* can decrease AFB1 concentration by 58% and 67% in 24 and 48 h, respectively, by converting the compound to AFR0 [7]. The yeast *Sacharomyces cerevisiae* can bind to AFB1 and reduce its levels in liquid cultures, although it is less effective than bacteria [8]. The fungi *Penicillium raistrickii*, *Rhizopus oligosporus*, and *Pleurotus ostreatus* [9] express laccase enzyme that degrades AFB1. Some aflatoxin-producing *Aspergillus* species are also capable of degrading their own synthesized mycotoxins [4].

Bacteria are the most important microorganisms that degrade aflatoxin, accomplished via adsorption, digestion, or degradation [4]. Different species of bacteria such as *Bacillus licheniformis* [10], *B. subtilis* [11], *Mycobacterium fluoranthenorans*

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[12], *Myxococcus fulvus* [13], *Nocardia corynebacterioides* [14], *Flavobacterium aurantiacum* [15, 16], *Rhodococcus erythropolis* [17], and *Stenotrophomonas maltophilia* [18] have been identified as AFB1-degrading bacteria. Lactobacilli and lactic acid-producing bacteria can reduce AFB1 through acid production under fermentation conditions. The AFB1-degrading activity of *Lactobacillus rhamnosus* [19], *L. gasseri* [20], *L. casei* [21], and *L. acidophus* [22] has also been demonstrated, while the AFB1-degrading activity of *F. aurantiacum* is influenced by various factors such as the presence of metal ions in the culture medium [15, 16].

AFB1 contamination is a major factor limiting the production and export of products such as pistachio in Iran [23]. Recent studies on AF biodegradation have mostly focused on the applicability of lactobacilli to animal husbandry and milk production; only one study has investigated the strength of aflatoxin degradation in the plant-bacterium *B. subtilis* [11].

In the present study, we isolated AFB1-degrading bacteria present in the garden and field soils of Birjand Plain in Eastern Iran—where important export crops such as pistachio, jujube, barberry, and medicinal plants are grown—in order to identify species potentially exploited for the purposes of AF decontamination.

MATERIALS AND METHODS

Reagents and Sample Collection

Standard solutions of AFB1 (Sigma-Aldrich, St. Louis, MO, USA) were prepared with methanol at a stock concentration of 10 ppm. Soil samples were collected from farms, gardens, and around municipal wastewater treatment centers in the Birjand plain during 2014–2015 for isolation of AFB1-degrading bacteria.

Bacterial Culture

Bacteria capable of growing on minimal medium [24] or de Man, Rogosa, and Sharpe (MRS) agar [25] containing 200 ppb of AFB1 were selected and further tested for AFB1-degrading activity. Selected isolates were cultured in nutrient broth (NB) or MRS broth (Merck Co.) for 12 h, and 1 ml of culture was transferred to NB or MRS broth (49 ml) in a flask and cultured at 37 °C with agitation for 24 h. A 1-ml volume of AFB1 solution (10 ppm) was added to the bacterial cultures for a final concentration of 200 ppb. Detoxification tests were carried out in the dark at 37 °C for 10 d [24]. After incubation, cells were

removed by centrifugation at 12000 rpm for 5 min and both cells and supernatant were examined. Sterile NB and MRSB were used as controls. Samples were extracted three times with chloroform according to Shotwell, Hesseltine [26] method and the extracts were evaporated under nitrogen gas. The residue was dissolved in 50% methanol in water (1:1, v/v) and analyzed using immunoaffinity column clean-up with HPLC and fluorescent detection [27] in TESTA Co. (Mashhad, Iran). The following formula was used to calculate the percentage of AFB1 degradation: $[\text{AFB1 peak area in control} - \text{AFB1 peak area in treated samples} / \text{AFB1 peak area in control}] \times 100$.

DNA Extraction and 16S Rrna Gene Amplification

Bacterial genomic DNA was extracted from AFB1-degrading bacteria using the cetyltrimethylammonium bromide method. The 16S rRNA gene was PCR-amplified with the 27f and 1492r general bacterial primers (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTACGACTT-3', respectively) [28]. PCR reactions were carried out using Taq PCR Master Mix (Sinaclon Co., Karaj, Iran) and included 12.5 µl Taq PCR Master Mix (0.2 U/µl Taq DNA polymerase in reaction buffer, 3 mM MgCl₂, and 0.4 mM each dNTP), 1 µl each 10 µmol/l primer, 1 µl fungal genomic DNA solution, and 9.5 µl distilled water in a total reaction volume of 25 µl [29]. Amplification conditions were as follows: 94 °C for 5 min; 35 cycles 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and 72 °C for 10 min [29]. Reaction products were analyzed on a 1% agarose gel stained with DNA Safe Stainv2 (Sinaclon Co.) and sequenced by Macrogen (Seoul, Korea). DNA sequences were manually edited using Bioedit v.7.1.3 and aligned using MEGA 6.0 software [30]. Phylogenetic analyses were carried out using MEGA 6.0 software [31] by the neighbor-joining method, and the degree of confidence in phylogenetic branching was assessed using 1000 bootstrap samples.

RESULTS

Afb1 Concentration in Bacterial Cultures

Of several bacterial strains from Gram-positive and negative isolated, five had AFB1-degrading activity. The AFB1 concentration in the control and other media before adding bacteria was 200 ppb. Samples were diluted by 1/20 for HPLC so that AF concentration in the analyzed control sample was 10 ppb. After the incubation period and analysis, the AF

concentration in the control medium was 9.02 ppb, representing a 0.98-ppb reduction, likely due to AF degradation during incubation. Since the solution was diluted by 1/20, after 10 d in the control medium, the concentration was 180.4 ppm; that is, 19.6 ppm was degraded over this period.

Identification of Afb1-Degrading Bacteria

The HPLC analysis revealed a range of AFB1 concentrations among samples. The AFB1 concentration in cultures of isolate F14—a Gram-positive endospore-forming species—was 1.03 ppm; the total amount of toxin in the culture medium was therefore 20.6 ppm, which was 151.8 ppm lower than that in control cultures. Thus, this isolate degraded 88% of AFB1 in the medium over a period of 10 d. A 1400-bp fragment corresponding to 16S rRNA was amplified from the genome of the F14 isolate (NCBI accession no. KX896754) and was found to have > 99% similarity to *B. pumilus* 16S rRNA.

Isolates F15 and F17 had the same biochemical characteristics of reducing AFB1 concentration in the medium. For F15, 1.9 ppm AFB1 was present in the solution analyzed by HPLC; therefore, the amount of toxin in the culture medium was 38 ppm, representing a decrease of 142.4 ppm relative to the control. Thus, this isolates degraded about 78% of

AFB1 in the medium (Fig. 1). The amount of AFB1 in the F17 culture medium was 3.5 ppm; after 10 d, the concentration was 70 ppm, indicating that this isolate degraded 61% of the toxin. Both strains were Gram-negative and oxidase positive. The 1281- and 1359-bp fragments amplified from isolates F15 and F17 (NCBI accession nos. KX896755 and KX896756, respectively) showed > 99.7% similarity to the *Ochrobactrum pseudogrignonens* 16S rRNA gene (Fig. 2).

Isolate F16 reduced AFB1 levels in the injected sample to 3.8 ppb after 10 d; thus, the toxin concentration was reduced from 180.4 to 76 ppm in the medium and 104 ppb AFB1 were degraded in 10 d, representing a decrease of 57%. F16 was a Gram-negative, facultative anaerobe; the amplified 1470-bp sequence (NCBI accession nos. KX896752) showed 100% similarity to the 16S rRNA sequence of *Enterobacter cloace*.

Isolate F18 reduced AFB1 concentration in the medium to 74 ppm during the analysis, corresponding to 58% degradation. This isolate was a Gram-negative, oxidase-positive, siderophore-forming bacterium; based on the 16S rRNA sequence (NCBI accession nos., KX896753) it was identified as *P. aeruginosa*.

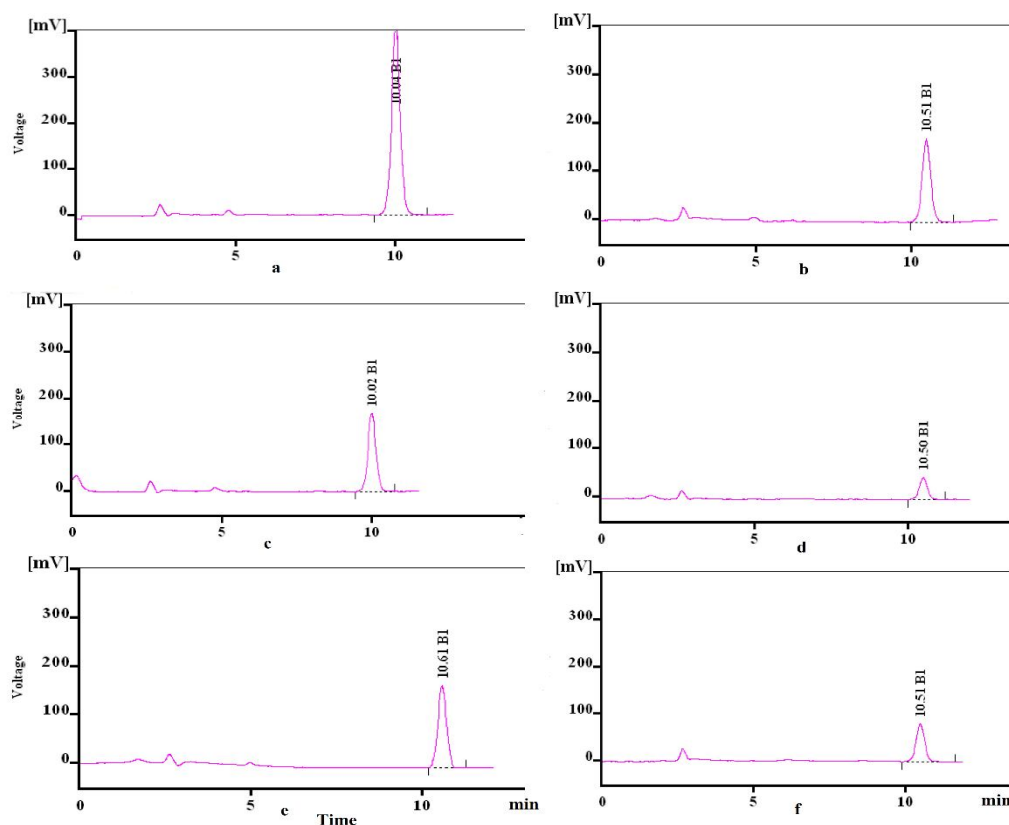


Figure 1. Chromatograms of AFB1 biodegrading bacteria after 10 days: (a) control; (b) *Pseudomonas aeruginosa*; (c) *Ochrobactrum pseudogrignonens* strain F18; (d) *Enterobacter cloace*; (e) *Bacillus pumilus*; (f) *O. pseudogrignonens* strain F15.

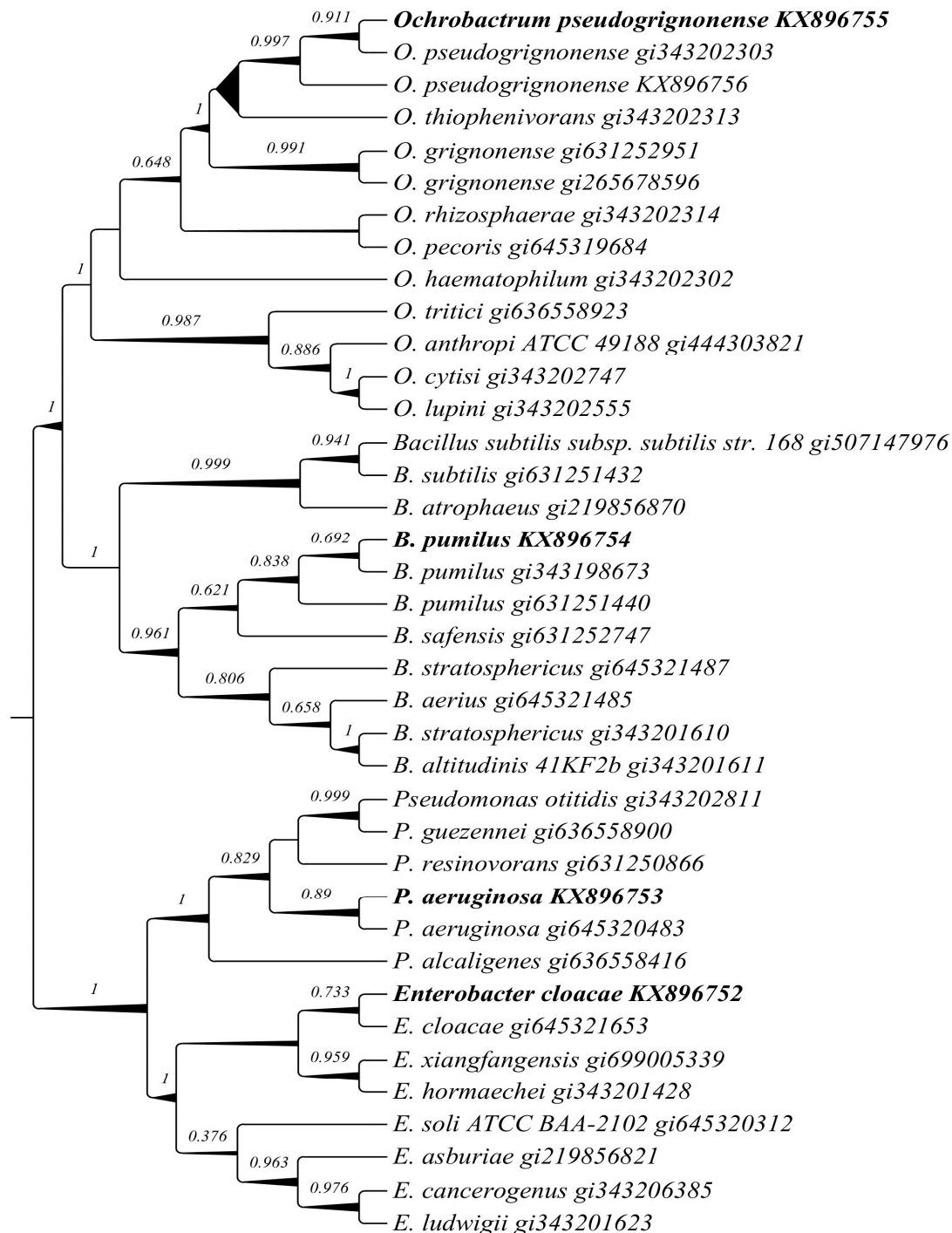


Figure 2. Neighbor-joining phylogenetic tree showing the relationships among AFB1 biodegrading strains based on partial 16S rRNA gene sequences (bold) Neighbor-joining distance tree was constructed with bootstrap values (% of 1,000 replicates).

DISCUSSION

Five bacterial strains from soil were collected in the Birjand plains that reduced AFB1 concentration in the culture medium by 57%–88% (102–160 ppb from 180.4 ppb) over a 10-day period. These strains were identified as *B. pumilus*, two isolates of *O. pseudogrignonens*, *P. aeruginosa*, and *E. cloacae*, which showed degradation activities of 88%, 78%, 61%, 58%, and 51%, respectively.

O. pseudogrignonens belongs to the Brucellaceae family in the Rhizobiales order (Kampfer, Scholz [32] isolated from the soil of wheat fields in France. We report for the first time the isolation of this species from a soil sample collected in Iran along with its capacity for AFB1 degradation. Our study also provides the first demonstration of AFB1 degradation by *B. pumilus*. Several species of the genus *Bacillus* are known to degrade AFB1 or the

structural analog coumarin; *B. subtilis* inhibited growth or AF production in *Aspergillus* species. A strain of *B. subtilis* was previously isolated in Thailand that suppressed *Aspergillus* growth and AF degradation capacity by up to 85% [10], while another study reported other strain of the same species that inhibited AFB₁, M-1, and J-1 degradation capacity by 60%–81% [33]. In Iran, a strain of *B. subtilis* isolated from pistachio trees was found to degrade > 90% of AF in liquid cultures at temperatures of 35 °C–40 °C over a period of 5 days [11]. In fact, the genus *Bacillus* had one of the highest capacities for degradation of chemical compounds, accomplished by production of extracellular enzymes [4]. Additionally, *Bacillus* species show a high degree of tolerance to adverse environmental conditions, especially in the desert where the temperatures and humidity cover broad ranges, suggesting greater ability to control plant pathogenic fungi and reduce AF contamination.

P. aeruginosa was also identified as an AFB₁-degrading bacterium. This species is found in soil and plant tissues or within the gastrointestinal tract of animals and can protect against certain plant diseases such as damping off. Only one previous report has described AFB₁, AFB₂ and AFM₁ degradation by this species isolated from corn by 82.8%, 46.8% and 31.9% after incubation in Nutrient Broth (NB) medium at 37 °C for 72 h, respectively [34]. We report here for the first time that *E. cloacae* can degrade AFB₁; this species is found in soil and plant tissues and has pathogenic potential in humans [35].

CONCLUSION

We identified five species of bacteria that have potent AFB₁-degrading activity. Although the precise mechanism remains to be determined, these findings provide a basis for the development of strategies that exploit these organisms for AFB₁ decontamination.

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REFERENCES

- Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Yoshinari T, Rezaee M-B, Jaimand K, Nagasawa H, et al. Inhibitory effects of *Satureja hortensis* L. essential oil on growth and aflatoxin production by *Aspergillus parasiticus*. *Int J Food Microbiol* 2008;123(3):228-33.
- Yoko I, Peterson SW, Donald T, Tetsuhisa G. *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section Flavi. *Mycol Res* 2001;105:233-9.
- Mace K, Aguilar F, Wang JS, Vautravers P, Gomez-Lechon M, Gonzalez FJ, et al. Aflatoxin B₁-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis* 1997;18:1291-7.
- Wu Q, Jezkova A, Yuan Z, Pavlikova L, Dohnal V, Kuca K. Biological degradation of aflatoxins. *Drug Metab Rev* 2009;41:1-7.
- Samarajeewa U, Sen A, Cohen M, Wei C. Detoxification of aflatoxins in foods and feeds by physical and chemical methods. *J Food Prot* 1990;53:489-501.
- Brown RL, Chen ZY, Cleveland TE, Russin JS. Advances in the Development of Host Resistance in Corn to Aflatoxin Contamination by *Aspergillus flavus*. *Phytopathology* 1999;89:113-7.
- Teunisson DJ, Robertson JA. Degradation of Pure Aflatoxins by *Tetrahymena pyriformis*. *Appl Microbiol* 1967;15:1099-103.
- Shetty PH, Hald B, Jespersen L. Surface binding of aflatoxin B₁ by *Saccharomyces cerevisiae* strains with potential decontaminating abilities in indigenous fermented foods. *Int J Food Microbiol* 2007;113:41-6.
- Alberts JF, Gelderblom WC, Botha A, van Zyl WH. Degradation of aflatoxin B(1) by fungal laccase enzymes. *Int J Food Microbiol* 2009;135:47-52.
- Petchkongkaew A, Taillandier P, Gasaluck P, Lebrihi A. Isolation of *Bacillus* spp. from Thai fermented soybean (Thua-nao): screening for aflatoxin B₁ and ochratoxin A detoxification. *J Appl Microbiol* 2008;104:1495-502.
- Farzaneh M, Shi Z-Q, Ghassempour A, Sedaghat N, Ahmadzadeh M, Mirabolfathy M, et al. Aflatoxin B₁ degradation by *Bacillus subtilis* UTBSP1 isolated from pistachio nuts of Iran. *Food Control* 2012;23:100-6.
- Hormisch D, Brost I, Kohring GW, Giffhorn F, Kroppenstedt RM, Stackebrandt E, et al. *Mycobacterium fluoranthenorans* sp. nov. a fluoranthene and aflatoxin B₁ degrading bacterium from contaminated soil of a former coal gas plant. *Syst Appl Microbiol* 2004;27:653-60.
- Zhao LH, Guan S, Gao X, Ma QG, Lei YP, Bai XM, et al. Preparation, purification and characteristics of an aflatoxin degradation enzyme from *Mycococcus fulvus* ANSM068. *J Appl Microbiol* 2011;110:147-55.
- Teniola OD, Addo PA, Brost IM, Farber P, Jany KD, Alberts JF, et al. Degradation of aflatoxin B(1) by cell-free extracts of *Rhodococcus erythropolis* and *Mycobacterium fluoranthenorans* sp. nov. DSM44556(T). *Int J Food Microbiol* 2005;105:111-7.

15. Lillehoj E, Ciegler A, Hall H. Aflatoxin B1 uptake by *Flavobacterium aurantiacum* and resulting toxic effects. *J Bacteriol* 1967;93:464-71.
16. Smiley R, Draughon F. Preliminary evidence that degradation of aflatoxin B1 by *Flavobacterium aurantiacum* is enzymatic. *J Food Prot* 2000;63:415-8.
17. Alberts JF, Engelbrecht Y, Steyn PS, Holzapfel WH, van Zyl WH. Biological degradation of aflatoxin B1 by *Rhodococcus erythropolis* cultures. *Int J Food Microbiol* 2006;109:121-6.
18. Guan S, Ji C, Zhou T, Li J, Ma Q, Niu T. Aflatoxin B1 degradation by *Stenotrophomonas maltophilia* and other microbes selected using coumarin medium. *Int J Mol Sci* 2008;9:1489-503.
19. Kankaanpää P, Tuomola E, El-Nezami H, Ahokas J, Salminen SJ. Binding of aflatoxin B1 alters the adhesion properties of *Lactobacillus rhamnosus* strain GG in a Caco-2 model. *J Food Prot* 2000;63:412-4.
20. Pierides M, El-Nezami H, Peltonen K, Salminen S, Ahokas J. Ability of dairy strains of lactic acid bacteria to bind aflatoxin M1 in a food model. *J Food Prot* 2000;63:645-50.
21. Hernandez-Mendoza A, Garcia H, Steele J. Screening of *Lactobacillus casei* strains for their ability to bind aflatoxin B1. *Food Chem Toxicol* 2009;47:1064-8.
22. Karunaratne A, Wezenberg E, Bullerman LB. Inhibition of mold growth and aflatoxin production by *Lactobacillus* spp. *J Food Prot* 1990;53:230-6.
23. Cheraghali AM, Yazdanpanah H, Doraki N, Abouhossain G, Hassibi M, Ali-abadi S, et al. Incidence of aflatoxins in Iran pistachio nuts. *Food Chem Toxicol* 2007;45:812-6.
24. De Bellis P, Tristezza M, Haidukowski M, Fanelli F, Sisto A, Mule G, et al. Biodegradation of Ochratoxin A by Bacterial Strains Isolated from Vineyard Soils. *Toxins* 2015;7:5079-93.
25. Bovo F, Franco LT, Rosim RE, Oliveira CAFd. Ability of a *Lactobacillus rhamnosus* strain cultured in milk whey based medium to bind aflatoxin B1. *Adv Food Sci Technol* 2014;34:566-70.
26. Shotwell OL, Hesseltine CW, Stubblefield RD, Sorenson WG. Production of aflatoxin on rice. *Appl Microbiol* 1966;14:425-8.
27. Pearson SM, Candlish AAG, Aidoo KE, Smith JE. Determination of aflatoxin levels in pistachio and cashew nuts using immunoaffinity column clean-up with HPLC and fluorescence detection. *Biotechnol Tech* 1999;13:97-9.
28. Li J-l, Wang S, Jing Y, Wang L, Zhou S-l. A modified CTAB protocol for plant DNA extraction. *Chinese Bulletin of Botany* 2013;48:72-8.
29. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, et al. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* 1998;64:795-9.
30. Gräfenhan T, Patrick SK, Roscoe M, Trelka R, Gaba D, Chan JM, et al. *Fusarium* damage in cereal grains from Western Canada. 1. Phylogenetic analysis of moniliformin-producing *Fusarium* species and their natural occurrence in mycotoxin-contaminated wheat, oats, and rye. *J Agric Food Chem* 2013;61:5425-37.
31. Tamura K, Stecher G, Peterson D, FilipSKI A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013;30(12):2725-9.
32. Kämpfer P, Scholz HC, Huber B, Falsen E, Busse H-J. *Ochrobactrum haematophilum* sp. nov. and *Ochrobactrum pseudogrignonense* sp. nov., isolated from human clinical specimens. *Int J Syst Evol Microbiol* 2007;57(11):2513-8.
33. Gao X, Ma Q, Zhao L, Lei Y, Shan Y, Ji C. Isolation of *Bacillus subtilis*: screening for aflatoxins B1, M1, and G1 detoxification. *Eur Food Res Technol* 2011;232:957-62.
34. Sangare L, Zhao Y, Folly YME, Chang J, Li J, Selvaraj JN, et al. Aflatoxin B1 degradation by a *Pseudomonas* strain. *Toxins* 2014;6:3028-40.
35. Sneath PH, Mair NS, Sharpe ME, Holt JG. *Bergey's manual of systematic bacteriology*. Williams & Wilkins; 1986.