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# Aflatoxin $B_1$ Binding by Microflora Isolated from Fermented Foods

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**Aflatoxins are major contaminants of wide range of food commodities which are susceptible to infection by *Aspergillus*, include cereals, oilseeds, spices and tree nuts. In the present study sixty eight lactic acid bacterial (LAB) cultures were isolated from fermented foods including *idli* batter, pickle, and fermented porridge (*koozhu*) and were screened for their ability to bind Aflatoxin $B_1$ ($AFB_1$). HPLC were used to quantify the aflatoxin during binding studies. Out of the 68 isolates tested, 45 isolates exhibited their ability to bind $AFB_1$ and 5 strains were able to bind more than 60% at 2 $\mu$g/ml concentration in Phosphate Buffer Saline (PBS) at pH 7. These five strains were *Leuconostoc lactis* (86.36%), *Lactococcus lactis* (78.7%), *Bacillus subtilis* (67.2%), *Pediococcus pentosaceus* (65.12%) and *Weissella confusa* (60.93%). This will help in the removal of $AFB_1$ naturally from food and feed by using these strains as the starter culture for fermented food.**

**Keywords:** Aflatoxin$B_1$; Aflatoxin binding; Fermented food; LAB.

Aflatoxins are group of important mycotoxins produced by *Aspergillus* sp. and *Penicillium* sp., known for their adverse effect on human and animal health (Lewis *et al*., 2005). Aflatoxins are grouped under class IA human carcinogens by International Agency for Research on Cancer (IARC, 1993). In view of the international concerns on the aflatoxins and their management, aflatoxins are one of the most important issues in the trade of various commodities between the nations (Kendra & Dyer, 2007). Aflatoxins can be produced at the time of pre or post-harvest as long as the favorable conditions exist (Peltonen *et al*., 2001). Various strategies have been developed for the management of aflatoxins in food and feed. However, it is very difficult for most of the strategies to removal of already produced aflatoxin in food and feed (Yiannikouris & Jouany, 2002; Méndez-Albores *et al*., 2007). The drawbacks of physical and chemical techniques for elimination, inactivation or reduction the bioavailability of aflatoxins such as loss of nutritional and safety qualities of the product as well as expensive equipments required for these techniques, have endorsed the recent prominence on biological methods (Teniola *et al*., 2005).

Some lactic acid bacteria (LAB) and yeast isolates has been reported to bind mycotoxins on their cell wall (Shetty & Jespersen, 2006) and the binding is specific and strain dependent (Shah & Wu, 1999). The most suitable candidate for aflatoxin binding is *Saccharomyces cerevicae* (Shetty & Jespersen, 2006; Shetty *et al*., 2007). Some probiotic strains are very effective in binding AFB1 with more than 80% of the toxin (Huskard *et al*., 1998). *Invitro* binding of AFB1 by LAB is described by fast and reversible process (Bueno *et al*., 2006) and strain and dose dependant (Kankaanpaa *et al*., 2000). Bueno *et al*., (2006) suggested mathematical model, in which mainly two processes

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(adsorption and desorption) are involved between bacterial surface and toxin. The alteration of bacterial surface by treatment with heat and acid increased the binding of aflatoxin (El-Nezami *et al*., 1998a). The bacterial strains and environmental conditions have been shown to be the key factor to stabilize the complexes formed between mycotoxins and LAB (Haskard *et al*., 2001). Non-viable LAB bound aflatoxin more effectively than their viable counterparts at low pH and when they enter in gut at low pH (Gratz *et al*., 2004; Kankaanpaa *et al*., 2000). Therefore, oral admiration of such LAB could be advantageous for humans and animals to reduce the bioavability of mycotoxins in the intestine. This study aimed to investigate AflatoxinB1 binding capacity of lactic acid bacterial strains isolated from Indian fermented foods. This the first study deals with the reduction of aflatoxin from Indian fermented foods.

## MATERIALS AND METHODS

### Bacterial cultures

Sixty eight bacterial isolates were used in this study. Among the 68 bacterial isolates, 5 MTCC type cultures were procured from IMTECH, Chandigarh and 63 were isolated from various fermented foods including *idli* batter, *koozhu* and pickle. The isolated bacteria were characterized by morphological and biochemical methods as per Bergey's Manual of Systematic Bacteriology (Kandler & Weiss, 1984). The bacterial strains were further identified by molecular characterization using 16S rRNA sequencing and were maintained at – 40ÚC in glycerol stocks for longer storage. Bacterial strains were reviewed and sub cultured in nutrient and de Man, Rogosa and Sharpe (MRS) broth prior to binding study.

### Aflatoxin $B_1$ standard

$AFB_1$ standards were procured from Himedia, Mumbai and a stock solution was prepared by dissolving $AFB_1$ in HPLC grade methanol at 1mg/ml concentration and quantitative confirmation was done by standard AOAC (1990) method. Working standards were prepared at 1, 2 and 3µg/ml concentration for calibration cure.

### Aflatoxin $B_1$ binding assay and optimization

Cultures were incubated with shaking at 35 ÚC in nutrient and MRS broth for 12 h. Cells were collected by centrifugation (8000 x g, for 5 min) and washed twice with phosphate-buffered saline solution (PBS) at pH 7. Finally, bacterial pellets were resuspended in 10 ml of sterile PBS. Cells were washed in buffer and adjusted to $10^9$ cells/ml concentration. One micro litre of cell suspension was centrifuged at 5000 x g for 5 min and the supernatant was removed completely. To the cell pellet, 950 ìl of PBS (pH 7) and 50 ìl of $AFB_1$ at 2, 5 and 10 µg/ml concentration and mixed thoroughly and incubated at 37°C at 200 rpm for 2, 6 and 12 h with the cell pellet from various growth stages. Tubes were centrifuged after incubation, at 10,000 x g for 10 min and the supernatant were analyzed for $AFB_1$ by HPLC (El-Nezami *et al*., 1998b).

### Quantification of unbound $AFB_1$ by HPLC

Quantification of $AFB_1$ in supernatants was done as described by Peltonen *et al*. (2001). The HPLC system (Shimadzu 20A, Tokyo, Japan) consisted of gradient pump (LC-20A), C18 column (250x 4.6mm, 5u Shiseido, Japan) and PDA detector (Shimadzu SPD-M20A) was used. A 20 µl sample was injected via auto injector (SIL-20AHT), micro filtered methanol- acetonitrile (40:60 v/v) was used as gradient mobile phase with a flow rate of 1.0 mL/min at 35 °C. $AFB_1$ detection was accomplished by PDA detector at 365 nm. Retention time of $AFB_1$ was approximately 2.92 min. The percentage of $AFB_1$ bound by the bacterial suspension was calculated using the following formula:

$$\%\ \text{AFB1} = \frac{[1 - (\text{AFB1 peak area of sample})]}{\text{AFB1 peak area of toxin control}} \times 100$$

### Statistical analysis

All the experiments were carried out at in triplicates and the experiments were repeated separately to confirm reproducibility.

## RESULTS AND DISCUSSION

### Screening for $AFB_1$ binding isolates from fermented foods

A total of 63 bacterial isolates from various fermented food sources along with 5 MTCC strains were screened for $AFB_1$ binding and degradation. Out of 63 isolates screened, 47 bacterial isolates were originated from *idli* batter, remaining 16 from brine pickle (10) and *koozhu* (6). All the tested cultures were able to bind $AFB_1$ and the level of binding appears to vary between the strains indicating the strain dependent nature of binding.

Five strains had been found more than 50% binding of the added $AFB_1$ with maximum binding up to 86.6 percent. 42 isolates bound more than 5% (Table:1), whereas the remaining 21 isolates bound less than 5% (data not shown). The five MTCC type cultures were showed 2.8 to 15.6 % binding (Table: 2). The *Leuconostoc lactis* (KC117496), *Lactococcus lactis* (KC834394), *Bacillus subtilis* (KC855550), *Weissella confusa* (KC895870) and *Pediococcus pentosaceus* (KF196839) from *idli* batter (with maximum binding ability) were selected for further optimization of $AFB_1$ binding. A number of strategies including physical, chemical and biological methods have been practiced to remove,

**Table 1.** Aflatoxin binding (%) of bacterial strains more than 5%

| S. No. | Culture NO. | Strain name | $AFB_1$ Binding (%) |
|---|---|---|---|
| 1 | PUFSTFMId34 | *Bacillus tequilensis* | 5.26±2.24 |
| 2 | PUFSTFMId17 | *Chryseobacterium vietnamense* | 5.31±2.09 |
| 3 | PUFSTFMId16 | *Bacillus subtilis* | 5.68±1.45 |
| 4 | PUFSTFMId30 | *Bacillus subtilis* | 5.89±2.48 |
| 5 | PUFSTFMId14 | *Bacillus amyloliquefaciens* | 6.23±2.67 |
| 6 | PUFSTFMId20 | *Acinetobacter baumannii* | 6.24±2.5 |
| 7 | PUFSTFMId51 | *Pediococcus pentosaceus* | 6.71±2.05 |
| 8 | PUFSTFMId45 | *Weisella confusa* | 6.91±1.71 |
| 9 | PUFSTFMId31 | *Bacillus subtilis* | 6.94±2.11 |
| 10 | PUFSTFMId18 | *Chryseobacterium vietnamense* | 7.02±1.41 |
| 11 | PUFSTFMId09 | *Bacillus tequilensis* | 7.09±2.7 |
| 12 | PUFSTFMId04 | *Bacillus cereus* | 7.57±2.21 |
| 13 | PUFSTFMId13 | *Bacillus subtilis* | 7.67±1.56 |
| 14 | PUFSTFMId42 | *Weisella confusa* | 7.9±2.03 |
| 15 | PUFSTFMId32 | *Bacillus tequilensis* | 8.83±1.30 |
| 16 | PUFSTFMId29 | *Bacillus safensis* | 9.07±2.5 |
| 17 | PUFSTFMId19 | *Bacillus tequilensis* | 9.17±2.07 |
| 18 | PUFSTFMId43 | *Weisella confusa* | 9.44±1.94 |
| 19 | PUFSTFMId08 | *Bacillus subtilis* | 9.78±2.32 |
| 20 | PUFSTFMId12 | *Bacillus subtilis* | 9.91±3.06 |
| 21 | PUFSTFMId10 | *Bacillus subtilis* | 10.12±3.52 |
| 22 | PUFSTFMId28 | *Weisella confusa* | 10.54±2.46 |
| 23 | PUFSTFMId38 | *Staphylococcus homonis* | 10.58±2.27 |
| 24 | PUFSTFMId11 | *Bacillus amyloliquefaciens* | 11.7±4.35 |
| 25 | PUFSTFMId54 | *Weisella ciberia* | 11.59±1.78 |
| 26 | PUFSTFMId40 | *Weisella confusa* | 12.08±2.59 |
| 27 | PUFSTFMId02 | *Bacillus subtilis* | 12.34±2.33 |
| 28 | PUFSTFMId06 | *Eterobacter cloacae* | 12.5±1.22 |
| 29 | PUFSTFMId26 | *Weisella confusa* | 14.29±3.19 |
| 30 | PUFSTFMId39 | *Weisella confusa* | 14.33±1.77 |
| 31 | PUFSTFMId15 | *Bacillus subtilis* | 14.55±1.89 |
| 32 | PUFSTFMId46 | *Micrococcus luteus* | 15.04±4.65 |
| 33 | PUFSTFMId05 | *Weisella confusa* | 16.13±3.24 |
| 34 | PUFSTFMId33 | *Lactococcus lactis* | 17.19±3.21 |
| 35 | PUFSTFMId03 | *Weisella confusa* | 18.12±4.1 |
| 36 | PUFSTFMId21 | *Weisella ciberia* | 19.12±3.84 |
| 37 | PUFSTFMId44 | *Weisella confusa* | 20.14±2.55 |
| 38 | PUFSTFMId52 | *Pediococcus pentosaceus* (*KF196839*) | 58.22±2.41 |
| 39 | PUFSTFMId41 | *Weisella confuse* (*KC895870*) | 60.93±2.45 |
| 40 | PUFSTFMId35 | *Bacillus subtilis* (*KC855550*) | 64.05±1.43 |
| 41 | PUFSTFMId24 | *Lactococcus lactis* (*KC834394*) | 74.56±1.06 |
| 42 | PUFSTFMId01 | *Leuconostoc lactis* (*KC117496*) | 78.96±2.55 |

inactivate or reduce the bioavailability of aflatoxins. Probiotics, when taken orally at adequate numbers, show beneficial effects on the host organism (Joint FAO/ WHO, 2002; CAST, 2007) and are able to reduce the bioavailability of consumed aflatoxins.

Several LAB from different sources are commonly consumed and characterized as Probiotic organisms. Previous investigation into $AFB_1$ binding by *Lactobacillus* sp. and *L. casei* has reported and the values ranging from 5% to 84% (Bolognani *et al*., 1997; El-Nezami *et al*., 1998b; Peltonen *et al*., 2000, 2001; Haskard *et al*., 2001; Lahtinen *et al*., 2004; Hwang *et al*., 2005; Zinedine *et al*., 2005). However our results have shown new finding in term of source of bacteria which was not reported earlier. The screening of 63 strains isolated from fermented foods which have wide range of binding capability in strain specific manner. The strain specific binding attribute is yet matter of investigation. Mathematical model proposed by Bueno *et al*., (2006) have indicated that differences in the types, numbers, and/or availability of $AFB_1$ binding sites have influence the binding ability. According to Lahtinen *et al*. (2004), cellular components mainly peptidoglycan, cell wall polysaccharides and proteins play a key role in surface binding of aflatoxins. Haskard *et al*. (2001) and Peltonen *et al*. (2001) have been suggested that the surface binding of $AFB_1$ is due to weak, non-covalent interactions, such as association with hydrophobic pockets present on the bacterial surface. Teichoic acids also play key role on $AFB_1$ binding by bacteria (Hernandez-Mendoza *et al*., 2009). However, $AFB_1$ binding is complex phenomena in which multiple components are also involved in the $AFB_1$ binding (Turbic *et al*., 2002). Additionally, environmental conditions can affect the interaction between bacterial surface and the toxin. $AFB_1$ binding in different conditions such as temperatures, pH, $AFB_1$ concentration, duration of exposure and cell density were analyzed in order to optimize the $AFB_1$ binding.

**Optimization of $AFB_1$ binding**

**Effect of growth stage**

Cells were collected at various growth stages (early and late exponential phases and stationary phase). The maximum binding was observed at early log phase at OD 0.5 (Fig. 1).

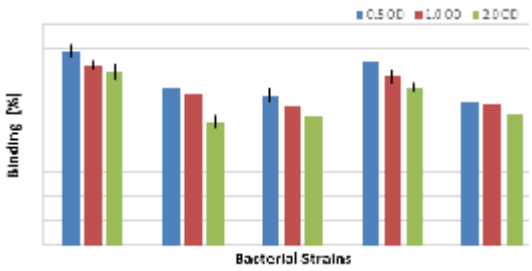


**Fig. 1.** Aflatoxin binding (%) of bacterial strains on different O.D value (0.5, 1.0 & 2.0)

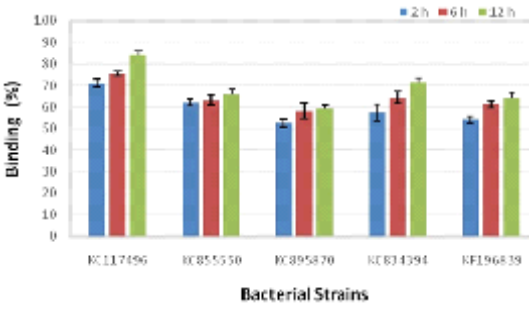


**Fig. 2.** Aflatoxin binding (%) of bacterial strains at different incubation period (2h, 6h & 12h) at OD 0.5

**Table 2.** Aflatoxin binding (%) of MTCC strains

| No. | MTCC strains | % of binding |
|---|---|---|
| 1. | *Lactobacillus acidophilus* (10307) | 2.86±1.54 |
| 2. | *Lactococcuslactis* (3041) | 7.49±3.12 |
| 3. | *Leuconostocmesentoid* (10508) | 10.31±4.17 |
| 4. | *Lactobacillus plantarum*(9495) | 13.24±2.6 |
| 5. | *Lactobacillus fermentum* (9748) | 15.62±3.25 |

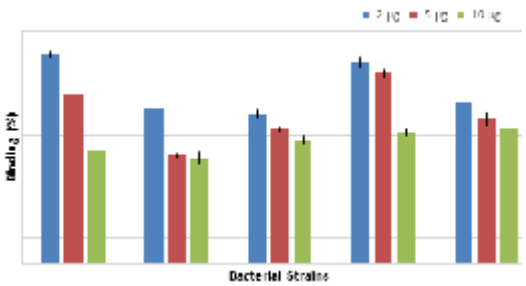


**Fig. 3.** Aflatoxin binding (%) of bacterial strains on different concentrations of Aflatoxin (2 µg, 5 µg & 10 µg) at OD 0.5 and incubation time 12 h

Binding ability decreased as cells grown older. It has been previously evidenced that bacterial concentration was playing major role in aflatoxin binding. According to El-Nezami *et al*., 1998b and Bolognani *et al*., 1997, a minimum of 2–5 x $10^9$ CFU/ml is required for significant $AFB_1$ removal (13–50%), and increase in cell concentration to 2 x$10^{10}$ CFU/ml was more effective (87 to 99% removal).

**Effect of exposure time**

In order to understand the effect of duration of exposure on binding, cells collected at OD 0.5 were incubated for various time periods with $AFB_1$. More than 50 % toxin was binding within 2 hours of exposure. The maximum binding level was observed at 12 h incubation (Fig. 2). It has showed that binding is fast process. Bueno *et al*., 2006, has reported that *in vitro* binding of aflatoxin is a fast and reversible process. So, after an optimal time reaction between $AFB_1$ and binding site was reached equilibrium. After that binding as well as release of $AFB_1$ occurs and there was no significance changes in binding percentage after a 12 h.

**Effect of $AFB_1$ concentration**

Figure-3 shows the effect of concentration of aflatoxin on binding. Optimal binding $AFB_1$ was observed at 2 µg per ml concentration at OD 0.5 and 12 h incubation time. With increasing concentration of $AFB_1$, $AFB_1$ removal was increased but there was no significant change in percentage (El-Nezami *et al*., 1998b). The method of validation was based on the previous study (El-Nezami *et al*., 1998b; Peltonen *et al*., 2001) with slight change. The optimum conditions were found to be 37 ºC, pH 7, 2µg $AFB_1$ concentration, 12 h interaction and a cell density of 0.5 OD.

## CONCLUSION

The results in the present study support the conclusions of preceding researchers that the ability of bacterial cells to bind $AFB_1$ and the stability of the bacterial cell–$AFB_1$ complex are strain dependent traits. This study reinforces the significance of removing mutagenic and carcinogenic compounds by binding assays under physiologically relevant conditions. It is particularly important to elucidate the interaction of intestinal and Probiotic bacteria with dietary mutagens and carcinogens and to assess the interactions of diet within the intestinal tract. The present study further supports the observation that species Probiotic lactic acid bacteria are able to bind dietary mutagens and carcinogens. The proposition that the bacteria are able to sequester the toxin and remove it from the gastrointestinal tract may partially explain the antimutagenic and anticarcinogenic effect of probiotics. The application of this phenomenon in the removal of mycotoxins from contaminated food and feed is urgently needed to improve the safety of food and feed supply.

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