Detoxification of AFM₁ in yogurt using *Lactobacillus casei*

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**ABSTRACT**

Yogurt is a fermented dairy product, manufactured by starter culture and consumed widely around the world. It may become contaminated with aflatoxin M₁ (AFM₁) that causes threats to the health of consumers, especially young children and adults. There are different methods to detoxify foods from AFM₁, but in yogurt, the easiest way is bio-detoxification method using different cultures and probiotic agents. So the aim of this study was to evaluate the effect of starter culture and *Lactobacillus casei* in detoxification of AFM₁. For this purpose skim milk powder was contaminated artificially with AFM₁ at levels: 0.05, 0.1, 0.5, and 0.75 ppb. Yogurt samples including control (inoculation just by starter culture-YC280) and treatments (inoculation by starter culture and *Lb.*casei-431) fermented at 42°C to reach pH<4.6 and consequently the AFM₁ content was measured by ELISA technique. Results showed that in the control samples and treatments, the toxin was removed 94.35 and 94.15 respectively. There was no significant difference between control and the treatment.

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**KEYWORDS**  
Aflatoxin M₁;  
Probiotic;  
Starter culture;  
Yogurt;  
*Lb.*Casei.

**INTRODUCTION**

Mycotoxins are secondary metabolites of molds, which are associated with certain disorders in animals and humans. In addition to being acutely toxic, some mycotoxins are now linked with the incidence of certain types of cancer, and it is this aspect that has evoked global concern over feed and food safety, especially for milk and milk products³. Aflatoxin M₁ (AFM₁) is a hepato-carcinogenic agent found in the milk of animals that have consumed feeds contaminated with aflatoxin B₁ (AFB₁), the main metabolite produced by the fungi of the genus Aspergillus, particularly *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius⁴*. About 0.3–6.2% of AFB₁ in animal feed is transformed to AFM₁ in milk Creppv⁶. Since AFM₁ has been evaluated as a possible human carcogen, the cancer risk arising from AFM₁ contamination in milk is a serious problem in food safety⁵. The occurrence of AFM₁ in milk, especially cow’s milk, makes it a particular risk factor for humans because of its importance as a foodstuff for adults and especially for children⁶. Due to serious health concerns, many countries have set maximum limits for aflatoxins, which vary from country to country⁴. The European Community prescribes that the maximum level of AFM₁ in liquid milk should not exceed 0.05 ppb.
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However, according to the US standard, the level of AFM$_1$ in liquid milk should not be higher than 0.5 ppb$^{[29]}$. Various physical and chemical methods have been used to detoxify AFs from food and 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$^{[9,10,17,37]}$. Bacteria like lactobacillus strains have been tested on their ability to inactivate AFs$^{[12]}$. In vitro studies have reported that bacterial concentration influences the AFB$_1$ removal. The binding mechanisms are yet not well understood, but a comparison between the removal ability of viable and nonviable bacteria has been previously reported, with nonviable bacteria providing the most effective removal, which suggests that AFB$_1$ reduction seems to be mainly by cell binding rather than metabolism or degradation$^{[28,26]}$. These trials have shown that the binding ability is strain-dependent. Lactobacillus casei strains have previously exhibited high affinity for binding AFM$_1$ in model systems$^{[13-15]}$. The aim of this study was to determine the efficacy of yoghurt starter culture and Lactobacillus casei to detoxify AFM$_1$ in yoghurt.

**MATERIALS AND METHODS**

**Materials**

**Culture production**

Counts of *Lb. casei* were enumerated according to Tharmaraj and Shah$^{[33]}$. MRS vancomycin agar was used for the enumeration of L. casei. The MRS broth was prepared according to manufacturer’s directions (EM Science Gibbstown NJ). Then about 2mL of 0.05 g vancomycin/100mL solution (Sigma Aldrich Co. St. Louis, MO) was added to 1000 mL of MRS broth to obtain 1 mg/L final concentration. Agar (EMD Chemicals, Gibbstown, NJ) was added at a concentration of 12 g/L. Final pH adjusted to 5.60. Yoghurts were sampled at weeks 1, 3 and 5 of storage. With a sterile pipette the yogurt in the cup was briefly agitated and 1 g yogurt was pipetted from the center of the yogurt cup into a sterile bottle containing 99mL of sterile peptone water (Difco, Detroit, MI). Contents were agitated, 10-fold serial dilutions were prepared. Plate counts were determined by plating serial dilutions of yogurt in MRS vancomycin agar. Pour plates were incubated anaerobically at 37 °C for 72 h. White, shiny, smooth colonies of 1.0 mm diameter were counted.

**TABLE 1 : Counts of *Lb. casei* from yoghurt enumerated on several media (CFU)**

<table>
<thead>
<tr>
<th>Media</th>
<th>Plate counting(CFU/ml) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS-agar</td>
<td>20×10$^5$ 9×10$^6$ 3×10$^7$</td>
</tr>
<tr>
<td>MRS Vancomycin agar</td>
<td>13×10$^5$ 3×10$^6$ 2×10$^7$</td>
</tr>
</tbody>
</table>

$a$: Plate counting results are the means of two plate assays.

**Contamination of reconstituted skim milk and yoghurt production**

Yoghurt samples made from reconstituted skim milk which prepared using skim milk powder (Merck-Germany). Reconstituted skim milk was heated at 93°C for 3min, then cooled to 42°C for inoculation. This milk sample divided into two equal portions. One of the portions inoculated with 1% YC-280 starter culture (Chr. Hansen) containing Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus. Another portion inoculated with starter culture and also Lactobacillus casei-431 (Chr. Hansen) as the probiotic agent to qualify the detoxification efficiency. The inoculated samples were contaminated with different doses of AFM$_1$ (0.05, 0.1, 0.5, and 0.75 ppb) purchased from Merck-Germany. All the samples were incubated at 42°C for 4 h for fermentation and yogurt production.

**AFM$_1$ analysis**

Yoghurt samples were centrifuged (at 2000 for 10 min at 4°C) and the supernatant fluids were analyzed for AFM$_1$ residues using direct competitive Enzyme-Linked Immunosorbent Assay (dc-ELISA) method. The ELISA system (BioTek, USA) consisted of ELISA reader (model ELx808), ELISA washer (model ELx50) and the ELISA kit (Euro Proxima). In the direct competitive ELISA (dc-ELISA) assay, the 96-wells ELISA plate coated with anti-AFM$_1$ antibodies (clones G11, 6G4, and ATX2) was used. One hundred $\mu$L of the supernatant fluid was directly used per well. One hundred $\mu$L of the AFM$_1$ standard solutions (0.05, 0.1,
0.5, and 0.75 ppb) and test samples (100 μL/well) in duplicate were added to the wells of microtiter plate and incubated for 60 min at room temperature in the dark. After the washing steps, 100 μL of the enzyme conjugate was added and incubated for 60 min at room temperature in the dark. The washing step was repeated three times. Fifty μL of substrate and 50 μL of chromogen were added to each well and mixed thoroughly and incubated for 30 min in the dark. Following the addition of 100 μL of the stop reagent to each well, the absorbance was measured at 450 nm in ELISA reader. According to the Euro-proxima kit guidelines, the lower detection limit is 6 ppt for milk.

**Statistical analysis**

The variance analysis was done for determining the difference between binding amounts of AFM$_1$ in the two media by ANOVA test by SPSS software version 15.1 at 95% level. All treatments were done in duplicate.

**RESULTS AND DISCUSSION**

The standard solutions of concentration from 0.05 to 0.75 ppb AFM$_1$ were used to find calibration/standard curve. The results showed the linearity of the standard curve over the range studied. Figure 1 gives the calibration curve of standard solutions of AFM$_1$ with concentrations of 0.05, 0.1, 0.5, and 0.75 ppb by ELISA analysis.

![Figure 1: Calibration curve of standard solutions of AFM1 with concentrations of 0.05, 0.1, 0.5, and 0.75 ppb by ELISA analysis](image)

**Effect of starter culture in detoxification of AFM$_1$**

The behavior of yoghurt starter culture (YC-280) in bio-detoxification of different concentrations of AFM$_1$ is shown in TABLE 2. According to the results, the average reduction rate of the mycotoxin was 94.35±0.073 ppb. So the starter culture removed AFM$_1$ significantly. Different studies have been done previously on the effect of starter cultures in detoxification of AFM$_1$. Our findings were similar to those found in a previously performed study by Maria Helena Iha et al[16]. They showed about 94.0% reduction in AFM$_1$ content of milk after yoghurt production. Some authors reported no influence on AFM$_1$ content[2,30,31,35,36]. In contrast, Munksgaard et al[25]. Bakirci[1]. detected variable increases of AFM$_1$ content in yogurt related to the milk. Govaris et al[11]. Studied the stability of AFM$_1$ in yoghurt artificially contaminated with AFM$_1$, during storage for 4 weeks. They showed that fermentation to a pH of 4.6, did not reduced the toxin content significantly, but in yoghurts having a pH of 4.0, AFM$_1$ decreased significantly (p < 0.01) after the third and fourth weeks of storage. The authors concluded that the decrease of AFM$_1$ could be a function of the low pH (4.0). But Van Egmond et al[34] observed no reduction of AFM$_1$ in yogurt stored for 7 days at 7ºC.

Megalla and Hafez[20] observed complete transformation of AFB$_1$ to its hydroxy derivative AFB$_2$-A caused by the acids present in yogurt. Whereas Rasic et al[27] revealed a high reduction (up to 97%) of AFM$_1$ in yogurt and acidified milk. El Deeb et al[7] observed that enzymatic, microbial, and particularly acid coagulation caused degradation of AFM$_1$ in buffalo milk. Maryamma et al[18] reported a high reduction of AFM$_1$ in fermented goat milk. It is known that exposure of the aflatoxin molecule to strong acid, such as trifluoroacetic acid, can cause its acid-catalyzed hydration, leading, for example, from AFB$_1$ to AFB$_2$-A Cohen and Lapointe[5].

**Effect of Lb.casei-431 in AFM$_1$ detoxification**

The behavior of *Lb.casei*-431 in bio-detoxification of different concentration of AFM$_1$ is shown in TABLE 3. According to the results the average reduction rate of the mycotoxin was 94.15±0.131 ppb. Different studies have been done previously on the effect of other lactic acid bacteria and probiotic agents to qualify their detoxification rate of AFM$_1$ (TABLE 4). Pierides et al[26] showed that specific strains of lactic acid bacteria bind the potent toxin non-covalently. Decrease in AFM$_1$
levels might be attributed to factors such as low pH, formation of organic acids or other fermentation by-products, or even to the presence of lactic acid bacteria. The low pH during fermentation alters the structure of milk proteins such as the caseins leading to formation of yoghurt coagulum. The change in caseins structure during yoghurt production may affect the association of AFM\textsubscript{1} with this protein Pierides et al\cite{26}. 

**TABLE 2 : Effect of starter culture (YC-280) in reduction of AFM\textsubscript{1} in yoghurt (control)**

<table>
<thead>
<tr>
<th>AFM\textsubscript{1} concentration (ng ml\textsuperscript{-1})</th>
<th>Mean YC-280 SD</th>
<th>Upper bound</th>
<th>Lower bound</th>
<th>Sig concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/75</td>
<td>0/1</td>
<td>0/5</td>
<td>0/05</td>
<td></td>
</tr>
<tr>
<td>86/36\textsuperscript{a}</td>
<td>93/39\textsuperscript{b}</td>
<td>98/59\textsuperscript{c}</td>
<td>99/09\textsuperscript{d}</td>
<td>0/163</td>
</tr>
<tr>
<td>94/35\textsuperscript{a}</td>
<td>0/073</td>
<td>0/131</td>
<td>98/96</td>
<td>86/23</td>
</tr>
<tr>
<td>99/09</td>
<td>0/75</td>
<td>0/292</td>
<td>0/292</td>
<td>0/0001</td>
</tr>
</tbody>
</table>

All treatments were done duplicate & data with a superscript in common do not differ significantly (p<0.05)

**TABLE 3 : Effect of Lb.casei-431 in reduction of AFM\textsubscript{1} in yoghurt (treatments)**

<table>
<thead>
<tr>
<th>AFM\textsubscript{1} concentration (ng ml\textsuperscript{-1})</th>
<th>Mean Lb.Casei-431 SD</th>
<th>Upper bound</th>
<th>Lower bound</th>
<th>Sig concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/292</td>
<td>0/05</td>
<td>0/131</td>
<td>98/96</td>
<td>86/23</td>
</tr>
<tr>
<td>94/15\textsuperscript{a}</td>
<td>0/073</td>
<td>0/131</td>
<td>98/96</td>
<td>86/23</td>
</tr>
<tr>
<td>99/09</td>
<td>0/292</td>
<td>0/292</td>
<td>0/292</td>
<td>0/0001</td>
</tr>
</tbody>
</table>

All treatments were done duplicate & data with a superscript in common do not differ significantly (p<0.05)

**TABLE 4 : Studies on LAB potential for AFM\textsubscript{1} detoxification rate**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Method of detection</th>
<th>Dose of AFM\textsubscript{1}</th>
<th>Detoxification rate of AFM\textsubscript{1} (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>L.rhamnosus strain GG</td>
<td>HPLC</td>
<td>0.15(µg/ml)</td>
<td>50/7± 2/1</td>
<td>Pierides et al.(2000)</td>
</tr>
<tr>
<td>L.rhamnosus strain LC-705</td>
<td>HPLC</td>
<td>0.15(µg/ml)</td>
<td>46/3± 2/6</td>
<td>Pierides et al.(2000)</td>
</tr>
<tr>
<td>L.gasseri (ATTC 33323)</td>
<td>HPLC</td>
<td>0.15(µg/ml)</td>
<td>30/8± 5/8</td>
<td>Pierides et al.(2000)</td>
</tr>
<tr>
<td>L.acidophilus strain LA1</td>
<td>HPLC</td>
<td>0.15(µg/ml)</td>
<td>18/3± 4/0</td>
<td>Pierides et al.(2000)</td>
</tr>
<tr>
<td>L.rhamnosus strain 1/3</td>
<td>HPLC</td>
<td>0.15(µg/ml)</td>
<td>18/1± 1/2</td>
<td>Pierides et al.(2000)</td>
</tr>
<tr>
<td>L.delbrueckii subsp.bulgaricus CH-2</td>
<td>ELISA</td>
<td>10(ng/ml)</td>
<td>18/7± 0/5</td>
<td>Sarimehmetoglu et al.(2004)</td>
</tr>
<tr>
<td>Streptococcus thermophilus ST-36</td>
<td>ELISA</td>
<td>10(ng/ml)</td>
<td>29/42± 0/6</td>
<td>Sarimehmetoglu et al.(2004)</td>
</tr>
<tr>
<td>L.bulgaricus</td>
<td>ELISA</td>
<td>0.05(µg/l)</td>
<td>87/6</td>
<td>El Khoury et al.(2011)</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>ELISA</td>
<td>0.05(µg/l)</td>
<td>70</td>
<td>El Khoury et al.(2011)</td>
</tr>
<tr>
<td>L.casei-431</td>
<td>ELISA</td>
<td>0.05, 0.1, 0.5, 0.75(µg/l)</td>
<td>94/15</td>
<td>Current study</td>
</tr>
</tbody>
</table>

**Comparison of detoxification rate of starter culture and Lb.casei-431**

According to TABLE 5 there is no significant difference between the effect of starter culture (YC-280) and Lb.casei (431) in AFM\textsubscript{1} detoxification rate (p=0.299). This means that the intervention had no significant effect on AFM\textsubscript{1} detoxification.

**TABLE 5 : Comparison of starter culture YC-280 and Lb. casei-431 in reduction of AFM\textsubscript{1} in yoghurt**

<table>
<thead>
<tr>
<th>AFM\textsubscript{1} concentration(ng ml\textsuperscript{-1})</th>
<th>Type of starter</th>
<th>Sig Type of starter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/75</td>
<td>0/292</td>
<td>0/0001</td>
</tr>
<tr>
<td>86/30\textsuperscript{a}</td>
<td>0/17\textsuperscript{b}</td>
<td>98/55\textsuperscript{c}</td>
</tr>
<tr>
<td>94/35\textsuperscript{a}</td>
<td>94/15\textsuperscript{b}</td>
<td>0/106</td>
</tr>
</tbody>
</table>

All treatments were done duplicate & data with a superscript in common do not differ significantly (p<0.05)

**REFERENCES**