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Detoxification of AFM_1 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 M_1 (AFM_1) that causes threats to the health of consumers, especially young children and adults. There are different methods to detoxify foods from AFM_1, 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 AFM_1. For this purpose skim milk powder was contaminated artificially with AFM_1 at levels: 0.05, 0.1, 0.5, and 0.75 ppb. Yogurt samples including control (inoculation just by started culture-YC280) and treatments (inoculation by starter culture and *Lb.casei-431*) fermented at 42°C to reach pH<4.6 and consequently the AFM_1 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.

KEYWORDS

Aflatoxin M_1;
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 products[3]. Aflatoxin M_1 (AFM_1) is a hepato-carcinogenic agent found in the milk of animals that have consumed feeds contaminated with aflatoxin B_1 (AFB_1), the main metabolite produced by the fungi of the genus Aspergillus, particularly *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*[9]. About 0.3–6.2% of AFB_1 in animal feed is transformed to AFM_1 in milk Creppv[6]. Since AFM_1 has been evaluated as a possible human carcinogen, the cancer risk arising from AFM_1 contamination in milk is a serious problem in food safety[32]. The occurrence of AFM_1 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 children[21-24]. Due to serious health concerns, many countries have set maximum limits for aflatoxins, which vary from country to country[4]. 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 AFB_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 manufacture'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. Yogurts 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 ºC 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)

Media	Plate counting(CFU/ml) [a]		
MRS-agar	20×10^5	9×10^6	3×10^7
MRS Vancomycin agar	13×10^5	3×10^6	2×10^7

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 4ºC) 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 μL of the supernatant fluid was directly used per well. One hundred μL 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 60min at room temperature in the dark. After the washing steps, 100μL of the enzyme conjugate was added and incubated for 60min 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 30min 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.

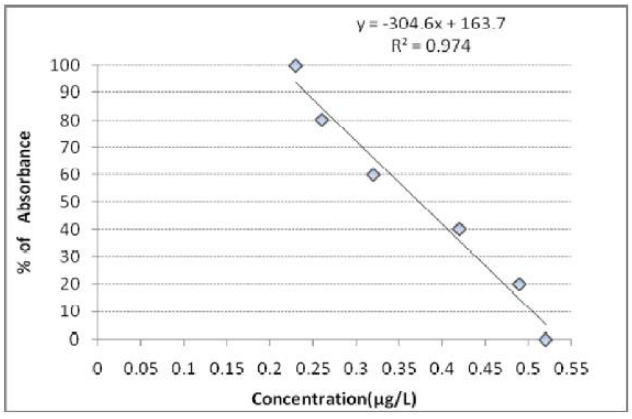

Figure 1 : Calibration curve of standard solutions of AFM1 with concentrations of 0.05, 0.1, 0.5, and 0.75 ppb by ELISA analysis

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 7 ºC.

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 trifluoracetic 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

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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_1 with this protein Pierides et al[26].

TABLE 2 : Effect of starter culture (YC-280) in reduction of AFM_1 in yoghurt (control)

	AFM_1 concentration (ng ml^{-1})				SD	Mean	SD	Upper bound	Lower bound	Sig
	0/05	0/1	0/5	0/75		YC-280				concentration
AFM_1 binding (%)	$86/36^a$	$93/39^b$	$98/59^c$	$99/09^d$	0/163	$94/35^a$	0/073	99/09	86/36	0/0001

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_1 in yoghurt (treatments)

	AFM_1 concentration (ng ml^{-1})				SD	Mean	SD	Upper bound	Lower bound	Sig
	0/05	0/1	0/5	0/75		Lb.Casei-431				concentration
AFM_1 binding (%)	$86/23^a$	$92/93^b$	$98/49^c$	$98/96^d$	0/292	$94/15^a$	0/131	98/96	86/23	0/0001

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_1 detoxification rate

Strain	Method of detection	Dose of AFM_1	Detoxification rate of AFM_1 (%)	References
L.rhamnosus strain GG	HPLC	0.15µg/ml	50/7± 2/1	Pierides et al.(2000)
L.rhamnosus strain LC-705	HPLC	0.15(µg/ml)	46/3± 2/6	Pierides et al.(2000)
L.gasseri (ATTCC 33323)	HPLC	0.15(µg/ml)	30/8± 5/8	Pierides et al.(2000)
L.acidophilus strain LA1	HPLC	0.15(µg/ml)	18/3± 4/0	Pierides et al.(2000)
L.rhamnosus strain 1/3	HPLC	0.15(µg/ml)	18/1± 1/2	Pierides et al.(2000)
L.delbrueckii subsp.bulgaricus CH-2	ELISA	10(ng/ml)	18/7± 0/5	Sarimehmetoglu et al.(2004)
Streptococcus thermophilus ST-36	ELISA	10(ng/ml)	29/42±0/6	Sarimehmetoglu et al.(2004)
L.bulgaricus	ELISA	0.05(µg/l)	87/6	El Khoury et al.(2011)
Streptococcus thermophilus	ELISA	0.05(µg/l)	70	El Khoury et al.(2011)
L.casei-431	ELISA	0.05, 0.1, 0.5, 0.75(µg/l)	94/15	Current study

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_1 detoxification rate (p=0.299). This means that the intervention had no significant effect on AFM_1 detoxification.

TABLE 5 : Comparison of starter culture *YC-280* and *Lb. casei-431* in reduction of AFM_1 in yoghurt

	AFM_1 concentration(ng ml^{-1})				SD	Type of starter		SD	Sig		
	0/05	0/1	0/5	0/75		YC-280	L.Casei -431		Conct	Type	Conct ×Type
AFM_1 binding (%)	$86/30^a$	$93/17^b$	$98/55^c$	$99/03^d$	0/167	$94/35^a$	$94/15^a$	0/106	0/0001	0/299	0/891

All treatments were done duplicate and data with a superscript in common do not differ significantly (p<0/05)

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