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Aflatoxin M_1 detoxification in kefir using *Lactobacillus acidophilus*

A.Mohammadi Sani[1], Z.Marhamati[2]*, M.H.Marhamatizade[3]

[1]Food Science and Technology Department, Quchan Branch, Islamic Azad University, Quchan, (IRAN)

[2]Food Science and Technology Department, Young Researchers Club, Quchan Branch, Islamic Azad University, Quchan, (IRAN)

[3]Food Hygiene Department, Veterinary Faculty, Kazerun Branch, Islamic Azad University, Kazerun, (IRAN)

E-mail: Zohreh.marhamati@yahoo.com

ABSTRACT

Kefir is fermented milk only made from kefir grains and kefir cultures and nowadays consumed widely around the world. It may become contaminated with aflatoxin M_1 (AFM_1) which even in small quantities, have hazardous effects for human beings. Therefore, a practical and effective method is needed to be developed for the detoxification of AFM_1 contaminated milk or decreased its toxicity. It has been reported that specific lactic acid bacteria are able to remove or degrade AFM_1 from liquid media by physical binding. The objective of this study was to detect the effect of kefir starter and *Lactobacillus acidophilus* to bind AFM_1 in kefir made from milk spiked with 500 pg AFM_1 mL^{-1}. Accordingly, five levels of kefir starter (2, 4, 6%, 8 and 10%) as group 1 and five levels of *Lb. acidophilus* (0.1, 0.3, 0.5, 0.7 and 0.9%) with constant amount of kefir starter (4%) as group 2 were used. After 48h, the AFM_1 content of kefir samples was measured by competitive ELISA technique. Statistical analyses, in group 1, showed that the sample containing 6% kefir starter had the most reduction in AFM_1 concentration (88.17%) which was significant ($p<0.05$). In group 2 the sample containing 0.9% *Lb. acidophilus* and 4% kefir starter had the maximum amount of AFM_1 binding (89.04%) and there were no significant differences ($p<0.05$) between 0.3, 0.5 and 0.7% levels in AFM_1 reduction. Generally, the effect of kefir starter (alone) was more than *Lb. acidophilus* in AFM_1 binding and the combination of these strains had synergistic effect in AFM_1 reduction. These findings affirmed that particular bacteria and yeast used in this study can offer decontaminating AFM_1 kefir.

KEYWORDS

Aflatoxin M_1;
Kefir starter;
Lb. acidophilus;
ELISA.

INTRODUCTION

Aflatoxins (AFs), a group of potent mycotoxins, are produced by some competent mould strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* as their secondary metabolic products[2,15,25]. These filamentous fungi easily occur on agricultural products during growth, harvest, storage or transportation and contaminated feeds and foods by producing toxins[13,19,32].

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Aflatoxin M_1 (AFM_1) is the monohydroxylated derivate of aflatoxin B_1 (AFB_1) which excreted into milk when lactating animals are feed with AFB_1 contaminated feeds[2,24]. It is estimated that approximately 0.3–6.2% of AFB_1 in animal feed is transformed to AFM_1 in milk, but this carryover rate has been shown to vary from animal to animal, day to day and also from one milking process to another. 12 h after ingestion of AFB_1 it could be revealed in milk and following the withdrawal of contaminated source, AFM_1 disappeared within 72 h[3,4,31].

Although, AFM_1, is less carcinogenic, hepatogenic and mutagenic than AFB_1, it can inhibit several metabolic systems and causing liver, kidney and heart damage[8,14,36]. So, occurrence of AFM_1 in milk and subsequently in other dairy products such as cheese, yogurt, butter, ice cream, kefir and etc. is a global concern since milk is a main nutrient for human diet particularly infants and children[7,18,34]. Due to serious health concerns, many countries have set maximum limits for aflatoxins, which vary from country to country[5,6]. The European Commission (EC) has set a limit of 50 ng/L for AFM_1 in milk[16] while the US food and drug administration[35] and institute of standards and industrial research of Iran[21] prescribed the maximum level for AFM1 500 ng/L.

The best way to control the presence of aflatoxins in foods and feeds is to prevent their formation. Various physical and chemical methods have been used to detoxify aflatoxins from food and feed materials. But the use of many of them is currently limited due to problems concerning safety[1,19,30]. This has led to search for alternative strategies such as biological agents. Studies undertaken in the last two decades suggested that lactic acid bacteria (LAB) and fermented dairy products possess anticarcinogenic activity. Recently, strains of LAB and yeasts were also reported to remove AFB_1 and AFM_1 from contaminated liquid media and milk[11,17,20,22,23,27,28]. This study focuses on the ability of kefir starter and *Lactobacillus acidophilus* for binding AFM_1 from kefir.

MATERIALS AND METHODS

Materials

Bacterial and yeast strains

Kefir starter (cominox company, Spain) and *Lactobacillus acidophilus* (CHR Hansen Company, Denmark) were used in this research.

Preparation of AFM_1 standard solution

AFM_1 powder (Sigma Chemical Co., St Louis, MO) was dissolved in a mixture of HPLC grade benzene/acetonitrile (97:3 v/v) to a concentration of 0.1µg/mL. Subsequently, the standard solution was prepared by diluting the mixture in PBS (0.5 M, pH 7.2). The benzene/acetonitrile was evaporated by heating (80 p C, 10 min) in a water bath[10,22]. The final concentration of the standard solution (0.05 µg/mL) was calculated using the Lambert-Beer equation ($A = \varepsilon. c. l$) using the absorbance at 450 nm. The resulting solution was transferred to a glass bottle and stored in the dark at 4°C until used[9,22].

Contamination of low-fat sterilized milk and kefir production

Five mL AFM_1 standard solution (0.05 µg/mL) was re-suspended in 495 mL of low-fat sterilized milk (1.5% fat), which was randomly purchased from a local supermarket in Shiraz-Iran, to a concentration of 500 pg of AFM_1 mL^{-1}. Kefir samples made from milk with AFM_1 (500 pg AFM_1/ml), as previously explained. In order to evaluate the ability of kefir starter and *Lb. acidophilus*, ten tubes were considered which divided into two groups. Moreover, two tubes were considered as control samples. First group including five tubes each containing 10 mL of contaminated milk were considered. Different doses of kefir starter: 0.2, 0.4, 0.6, 0.8 and 1g (2, 4, 6, 8 and 10%) were added to the tubes respectively and mixed properly so that kefir starter was uniformly distributed. Second group including five tubes each containing 10 mL of contaminated milk and constant amount of kefir starter (0.4 gr which equals to 4%). *Lb. acidophilus* was added directly to all tubes in different concentrations: 0.01, 0.03, 0.05, 0.07 and 0.09 gr (0.1, 0.3, 0.5, 0.7 and 0.9%) and mixed properly. Afterwards, all the ten tubes were placed in incubator at 24°C for 24 h. Then coagulum was separated from the milk by filtering with a filter paper and the liquid was distributed in caps then placed in incubator at 14°C for 24 h. Finally, kefir samples were stored at 4°C in refrigerator for 48 h, then ELISA test procedure was performed. In order to prepare control samples two tubes (C_1 and C_2) were considered. C_1 sample

was for evaluating the initial contamination of low-fat sterilized milk which containing 10 mL of milk. It was stored at 4°C in refrigerator for 48 h, and finally ELISA test procedure was performed. C_2 sample containing 10 mL of contaminated milk plus 0.1% (0.01 g) *Lb. acidophilus* which was added directly and mixed properly. Then placed in incubator at 38°C for 8 h and finally stored at 4°C in refrigerator for 48 h. Afterwards, ELISA test procedure was performed.

AFM_1 analysis

AFM_1 analysis was performed by ELISA procedure according to EuroProxima B.V. recommendations. All samples were centrifuged (at 2000 for10 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 µL of the supernatant fluid was directly used per well. One hundred µL of the AFM_1 standard solutions 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. The liquid was poured off the wells and the micro well holder was tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. All the wells were filled with 250 µL of washing buffer and emptied as described earlier. The washing procedure was repeated twice. One hundred µL of the enzyme conjugate was added and incubated for 60 min at room temperature in the dark. The washing sequence was repeated three times. 50 µL of substrate and 50 µL of chromogen were added to each well and mixed thoroughly and incubated for 30 min at room temperature in dark. Then 100 µL of the stop reagent was added to each well, mixed, and measured at wave long of 450 nm in ELISA reader.

Statistical analysis

Statistical analyses of AFM_1 removal assays were carried out by using the Student's t-test for significant differences between binding amounts of AFM_1 by the two microorganisms at different levels (kefir starter and *Lb. acidophilus*). All treatments were done in duplicate.

RESULTS AND DISCUSSION

The optical density (OD) values of the standards and samples were divided by the mean OD value of the zero standard and multiplied by 100. The zero standard was thus made equal to 100% (maximal absorbance) and the other OD values were quoted in percentages of the maximal absorbance. The amount of AFM_1 in the samples was expressed as AFM_1 equivalents. The AFM_1 equivalents in the samples (pg/mL) corresponding to the maximal absorbance percentage of each extract can be read from the calibration curve (Figure 1). TABLE 1 shows the concentration of unbound AFM_1 in each sample according to amount of absorbance OD_{450nm} based on standard curve.

Effect of kefir starter in detoxification of AFM_1

The results for ability of different levels of kefir starter to bind AFM_1 are presented in Figure 2. AFM_1 levels in kefir samples treated with different doses of kefir starter ranged from 81.83 to 88.17%. Although all samples had more than 80% AFM_1 reduction after 48 h, the highest reduction of AFM_1 was related to the sample containing 6% kefir starter (88.17%) and then the samples containing 8, 10, 4 and 2% kefir starter had lower amounts of reduction, respectively. The percentages of AFM_1 binding in these four samples were

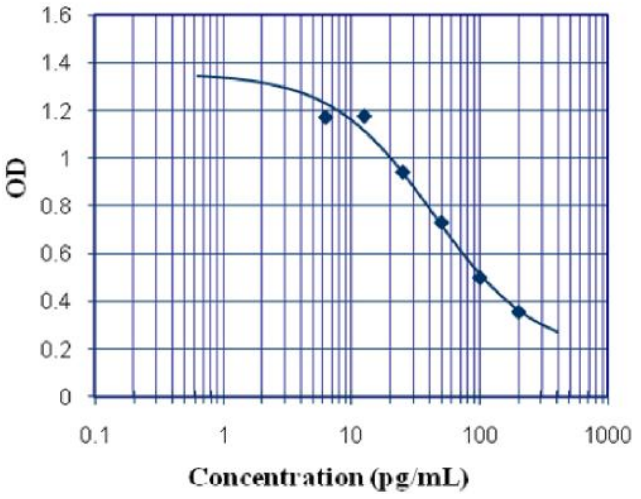

Figure 1 : Calibration/standard curve

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TABLE 1 : The concentration of unbound AFM_1 in each sample according to amount of absorbance OD_{450nm} based on standard curve

sample		AFM_1 added to milk ($pgmL^{-1}$)	Initial AFM_1 in milk ($pg\ mL^{-1}$)	Unbound AFM_1 in kefir ($pg\ mL^{-1}$)	AFM_1 absorbance (%)
C_1		-----	188	188	27.16
C_2		500	688	147	30.63
Kefir starter samples	T_1	500	688	125	33.28
	T_2	500	688	107	35.79
	T_3	500	688	81.4	37.49
	T_4	500	688	102	33.87
	T_5	500	688	107	33.21
Lb. acidophilus samples	T_6	500	688	102	36.61
	T_7	500	688	86.9	40.07
	T_8	500	688	83.9	40.96
	T_9	500	688	81.3	41.62
	T_{10}	500	688	75.4	43.47

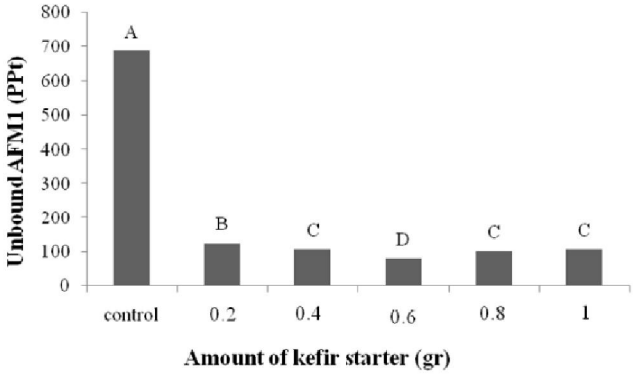

Figure 2 : Effect of kefir starter in reduction of AFM_1 in kefir

equal to 85.17, 84.45, 84.45 and 81.83%. No significant differences ($p<0.05$) were found between AFM_1 binding in treatments with 4, 8 and 10% of kefir starter. Consequently, it is suggested to use 6% kefir starter to achieve the maximum reduction of AFM_1 in industrial production of kefir. There is no previous report on using kefir starter to decontaminate kefir for AFM_1 (TABLE 2). The mechanism involved in kefir starter ability to bind aflatoxins remains unclear. It is currently accepted that yeast cell wall has the ability to absorb the toxin[11,12,26,27,33].

Effect of *Lb. acidophilus* in detoxification of AFM_1

Figure 3 shows the effect of *Lb. acidophilus* alone and in presence of kefir starter at different levels in AFM_1 reduction. After 48 h, the results of our study revealed that 0.1% *Lb. acidophilus* (without kefir starter) removed 78.63% of AFM_1 content. Using 0.1% *Lb. acidophilus* with a constant amount of kefir starter (4%) caused significant increase ($p<0.05$) in the percentage of AFM_1 binding which was equal to 85.17%. AFM1 levels in kefir samples treated with different doses of *Lb. acidophilus* and constant amount of kefir starter (4%) ranged from 85.17 to 89.04%.

The sample containing 0.9% *Lb. acidophilus* and 4% kefir starter had the maximum amount of AFM_1 binding (89.09%) and then the samples containing 0.7, 0.5, 0.3 and 0.1% starter had lower amounts of reduction, respectively. The percentages of AFM_1 binding in these four samples were equal to 88.18, 87.81, 87.37 and 85.17% respectively. Statistical analyses showed no significant differences ($p<0.05$) between 0.3, 0.5 and 0.7 levels in AFM_1 reduction.

Our results shows that by increasing *Lb. acidophilus* amount, AFM_1 binding increased and the sample containing 0.9% *Lb. acidophilus* and 4% kefir starter showed the maximum AFM_1 reduction. Generally, the

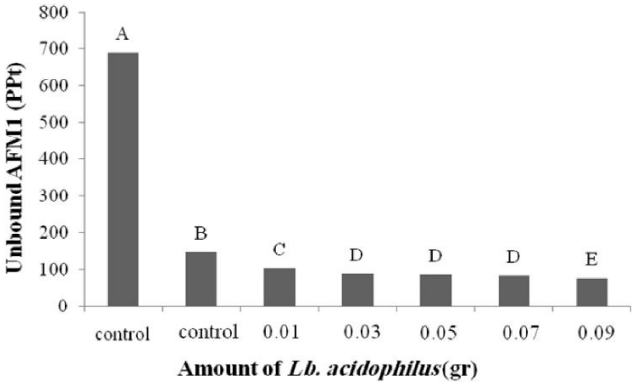

Figure 3 : Effect of *L. acidophilus* in reduction of AFM_1 in kefir

effect of kefir starter (alone) was more than *Lb. acidophilus* in AFM_1 binding and the combination of kefir starter and *Lb. acidophilus* had synergistic effect in AFM_1 reduction.

TABLE 2 : Studies on LAB and yeast potential for AFM_1 detoxification

Strain	Product type	Method of detection	AFM_1 concentration		AFM_1 Detoxification rate (%)		Reference
S. cerevisiae			Milk	HPLC	0.5 ng/mL	92.7 ± 0.7	Corrassin et al. (2013)
LAB pool [1]			Milk	HPLC	0.5 ng/mL	11.7 ± 4.4	Corrassin et al. (2013)
S. cerevisiae + LAB			Milk	HPLC	0.5 ng/mL	Nearly 100	Corrassin et al. (2013)
L. acidophilus Lf10			Yogurt	HPLC	5 µg/L	18.4 ± 0.5	Motawee et al. (2011)
Streptococcus thermophilus K45			Yogurt	HPLC	5 µg/L	28.2 ± 4.3	Motawee et al. (2011)
L. bulgaricus R21			Yogurt	HPLC	5 µg/L	31.4 ± 2.6	Motawee et al. (2011)
L. helveticus A34			Yogurt	HPLC	5 µg/L	29.4 ± 1.5	Motawee et al. (2011)
L. rhamnosus GG			Yogurt	HPLC	5 µg/L	48.4 ± 2.8	Motawee et al. (2011)
L. rhamnosus LC705			Yogurt	HPLC	5 µg/L	49.6 ± 2.4	Motawee et al. (2011)
L. bulgaricus			Yogurt	ELISA	0.05 µg/L	87.6	El khoury et al. (2011)
Streptococcus thermophilus			Yogurt	ELISA	0.05 µg/L	70	El khoury et al. (2011)
L. delbrueckii subsp. *Bulgaricus* CH-2			Yogurt	ELISA	10 ng/mL	18.7 ± 0.5	Sarimehmetoglu et al. (2004)
Streptococcus thermophilus ST-36			Yogurt	ELISA	10 ng/mL	29.42 ± 0.6	Sarimehmetoglu et al. (2004)
L. rhamnosus GG			Milk	HPLC	0.15 µg/L	50.7 ± 2.1	Pierides et al. (2000)
L. rhamnosus LC705			Milk	HPLC	0.15 µg/L	46.3 ± 2.6	Pierides et al. (2000)
L. gasseri			Milk	HPLC	0.15 µg/L	30.8 ± 5.8	Pierides et al. (2000)
L. acidophilus LA1			Milk	HPLC	0.15 µg/L	18.3 ± 4.0	Pierides et al. (2000)
L. rhamnosus strain 1/3			Milk	HPLC	0.15 µg/L	18.1 ± 1.2	Pierides et al. (2000)
C. kefir			Kefir	ELISA	0.5 ng/mL	85	Current study
L. acidophilus			Kefir	ELISA	0.5 ng/mL	78.63	Current study
L. acidophilus + *C. kefir*			Kefir	ELISA	0.5 ng/mL	87.51	Current study

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