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Bio-detoxification of aflatoxin M_1 in kefir using *Lactobacillus casei*

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ABSTRACT

Kefir is a fermented dairy product, manufactured by starter culture and nowadays consumed widely around the world. It may become contaminated with aflatoxin M_1 (AFM_1) which even in small quantities, have harmful effects on consumers health. Therefore, a practical and effective method is needed for detoxification of AFM_1 contaminated milk or decreasing its toxicity, such as using different cultures and probiotic agents. Specific lactic acid bacteria strains have been reported in removing AFM_1 from liquid media by physical binding. The aim of this study was to detect the effect of kefir starter and *Lactobacillus casei* to bind AFM_1 in kefir made from milk spiked with 500pg AFM_1 mL^{-1}. Accordingly, five levels of kefir starter (2%, 4%, 6%, 8%, 10%) and five levels of *Lb. casei* (0.1%, 0.3%, 0.5%, 0.7% and 0.9%) with constant amount of kefir starter (4%) were used, separately. After 48h, the AFM_1 content of kefir samples was measured by competitive ELISA technique. Statistical analyses showed that the sample containing 6% kefir starter had the most reduction in AFM_1 concentration (88.17%) which was significant ($p<0.05$). Although there were no significant differences ($p<0.05$) between AFM_1 concentration of samples containing 0.5, 0.7 and 0.9% levels of *Lb.casei*, but the sample containing 0.9% *Lb.casei* and 4% kefir starter, had more AFM_1 binding (82.12%). Generally, the effect of kefir starter (alone) was more than *Lb. casei* 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. casei;
ELISA.

INTRODUCTION

Mycotoxins are toxic substances produced by filamentous fungi that grow on agricultural products in the field during growth, harvest, storage and transportation[25,32]. Aflatoxins, a group of potent mycotoxins, are common contaminant of foods, especially in the staple diets of many developing countries[2,15]. There are currently 18 similar compounds described by the term aflatoxin, which are distinguished

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by their wide distribution in food, and pronounced toxic properties[13,29].

Aflatoxin M_1 (AFM_1) is the monohydroxylated derivate of aflatoxin B_1 (AFB_1), metabolized by cytochrome P450 enzyme system in liver and can be found in milk of lacting animals that have ingested feed contaminated with some common molds such as *Aspergillus flavus, Aspergillus parasiticus* or *Aspergillus nomius*[2,24]. About 0.3–6.2% of AFB_1 in animal feed is transformed to AFM_1 in milk, which could be revealed in milk within 12h after ingestion of AFB_1 and following the withdrawal of contaminated source, AFM_1 disappeared within 72h[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]. Thereupon 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 major constituent of 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 50ng/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 AFM_1 500ng/L.

Various physical and chemical methods have been used to reduce the aflatoxin level in foods and feeds for many years[19]. The use of many of the available physical and chemical methods for detoxification of contaminated products is restricted due to problems concerning safety issues and undesirable health effects, possible losses in nutritional quality of treated commodities, coupled with limited efficacy and cost implications[1,19,30]. This has led to search for alternative strategies such as biological agents. Particular Lactic acid bacteria (LAB) strains and yeasts have been tested for their ability to bind aflatoxins[11,17,20,22,23,27,28]. The removal of aflatoxin involves physical binding of the toxin probably to the bacterial cell wall or cell wall components[23]. The present work was conducted to study the ability of kefir starter and *Lactobacillus casei* for binding AFM_1 from kefir.

MATERIALS AND METHODS

Materials

Bacterial and yeast strains

Kefir starter (cominox compani, spain) and *Lactobacillus casei* JQ301798.1 GI372220084 (separated from dairy products) which registered in the National Center for Biotechnology Information advances science and health (NCBI) 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 4p 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 495mL 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. casei*, ten tubes were considered which divided into two groups. Moreover, two tubes were considered as control samples. First group including five tubes each containing 10mL of contaminated milk were prepared. Different doses of kefir starter 0.2, 0.4, 0.6, 0.8 and 1gr (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 10mL of contaminated milk and constant amount of kefir starter (0.4gr which equals to 4%). *Lb. casei* was added directly to all tubes in different concentrations: 0.01, 0.03, 0.05, 0.07 and 0.09 and mixed properly. Afterwards, all the ten tubes

were placed in incubator at 24p 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 14p C for 24 h. Finally, kefir samples were stored at 4p 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 for evaluating the initial contamination of low-fat sterilized milk which contained 10mL of milk. It was stored at 4p C in refrigerator for 48 h, and finally ELISA test procedure was performed. C_2 contained 10mL of contaminated milk plus 0.1% (0.01g) *Lb. casei* which was added directly and mixed properly. Then the tubes incubated at 38p C for 8h and finally stored at 4p C in refrigerator for 48h. 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 4æ%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 (100µL/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 250µL 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. Fifty µL of substrate and 50µL of chromogen were added to each well and mixed thoroughly and incubated for 30 min at room temperature in dark. Then 100µL 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. casei*). 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

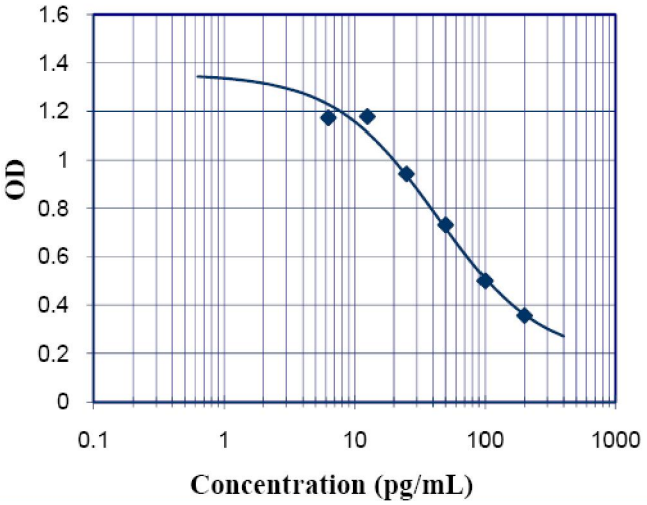

Figure 1: Calibration/standard curve

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 Fig 2. AFM_1 levels in kefir samples treated with different doses of kefir starter

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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	212	26.61
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
	T_6	500	688	102	36.61
Lb. casei samples	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

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 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].

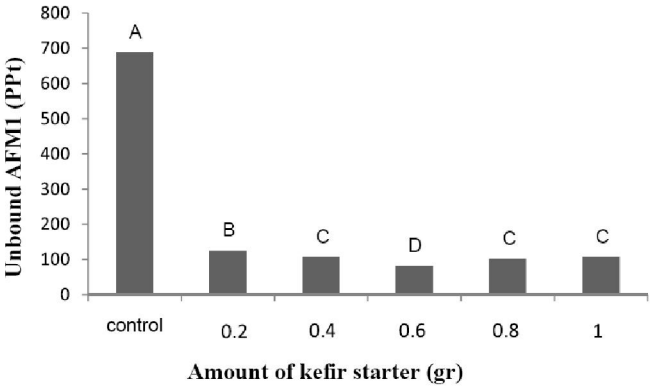

Figure 2 : Effect of kefir starter in reduction of AFM_1 in kefir

Effect of *Lb. casei* in detoxification of AFM_1

Figure 3 shows the effect of *Lb. casei* alone and in presence of kefir starter at different levels in AFM_1 reduction. After 48h, the results of our study revealed that 0.1% *Lb. casei* (without kefir starter) removed 69.19% of AFM_1 content. Using *Lb. casei* 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 81.40%. AFM_1 levels in kefir samples treated with different doses of *Lb. casei* and constant amount of kefir starter (4%) ranged from 81.40-82.12%. Although there were no significant differences ($p<0.05$) between treatments with 0.5, 0.7 and 0.9% levels, the sample containing 0.9% *Lb. casei* was more effective in AFM_1 reduction 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

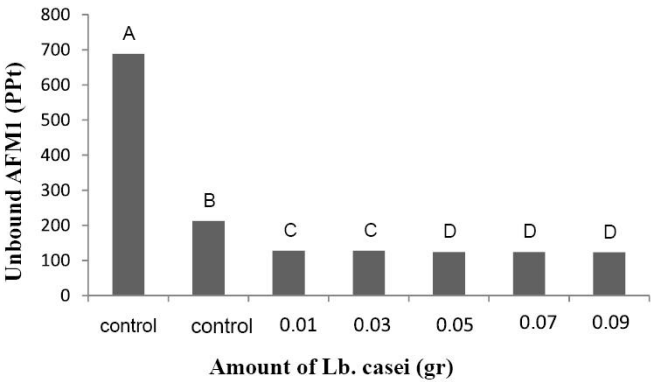

Figure 3 : Effect of L. casei in reduction of AFM_1 in kefir

equal to 81.98, 81.89, 81.40 and 81.40% respectively. Statistical analyses showed no significant differences ($p<0.05$) between 0.1 and 0.3% levels in amount of AFM_1 binding efficacy

Our results shows that by increasing *Lb. casei* amount, AFM_1 binding increased and the sample containing 0.9% *Lb. casei* and 4% kefir starter showed the maximum AFM_1 reduction. Generally, the effect of kefir starter (alone) was more than *Lb. casei* in AFM_1 binding and the combination of kefir starter and *Lb. casei* 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. casei	Kefir	ELISA	0.5 ng/mL	69.19	Current study
L. casei + C. kefir	Kefir	ELISA	0.5 ng/mL	81.76	Current study

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