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THE ASSESSMENT OF REDUCING AFLATOXIN M1 IN KEFIR BY SACCHAROMYCES *KEFIR* AND *LACTOBACILLUS CASEI TD4* BY ELISA METHOD

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ABSTRACT

Aflatoxin M_1 (AFM_1) is the metabolite of the aflatoxin B_1 (AFB_1) and highly toxic compound. Occurance of AFM_1 in milk and dairy products had hazardous effects for human beings especially children and elderly .It has been proposed that the consumption of lactic acid bacteria (LAB) and some of the yeast capable of binding or degrading food-borne carcinogens would reduce human exposure to these deleterious compounds. The purpose of this study was to evaluate the ability of *Candida kefir* strain and *Lactobacillus Casei* TD_4 to bind AFM1 and create different starters from these strains that have more ability to reduce AFM1 in kefir that made from milk spiked with AFM1. Accordingly, five levels of kefir starter 4, 8, 12, 16 and 20% were used. In addition, *L. casei* were used at five levels 0.1, 0.3, 0.5, 0.7 and 0.9% with constant amount of kefir starter (8%) separately. The amount of unbound AFM1 was analyzed by competitive ELISA. Results showed that all assessed strains whether *L. casei* or kefir starter exhibited different degrees of aflatoxin binding in kefir. The sample containing 20% kefir starter had the most reduction of AFM1 (91.91%) and between bacterial samples, *Lactobacillus Casei* TD_4 whit (*92.52 %)* at 0.9% level had the maximum amount of AFM1 binding. In general, When yeast was used with LAB the removal efficiency of AFM1 significantly increased. These findings supported that specific bacteria and yeast used in this study can offer decontaminating AFM1 kefir.

KEY WORDS: Aflatoxin M_1, *Candida kefir*, *Lactobacillus Casei* TD_4, ELISA

INTRODUCTION

Mycotoxins are food contaminants with detrimental effect on human and animal health. Mycotoxin contamination is a serious problem in the world and is especially widespread in developing countries. Aflatoxin is the most frequently found in human foods (El-Nezami et al. 1998; Fuchs et al. 2008). Aflatoxins are a group of secondary metabolites of fungi that grow on a variety of food and feed commodities at any stage of production. They have harmful effects on the health of

humans and animals, including carcinogenic, mutagenic, teratogenic, and immunosuppressive effects (yiannikouris and Jouany, 2002; Creppy, 2002; Razavilar, 2003) and also cause economic losses in industry due to the contamination of food and feed (Haskard et al. 2001). There have been identified 18 types of aflatoxins, nevertheless, the naturally occurring and well-known ones are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) (Gimeno, 2004; Saleemullah et al. 2006; Strosnider et al. 2006). AFM1 is a metabolic derivate of AFB1, and AFM2 is a metabolic derivate of AFB2; both come from the metabolism of some animals, and are normally found in milk, milk products and urine (hence the designation M1) (Strosnider et al. 2006; Boriboon and Suprasert, 1994; Prandini et al. 2009). Although AFM1 is about 10 times less cytotoxic, genotoxic and carcinogenic than AFB1, it can inhibit some metabolic systems and causing liver, kidney and heart damage. (Murphy et al. 2006). Aflatoxin may be degraded by physical, chemical or biological means (Nkana, 1987; Park, 1993). However, limitations such as losses of product nutritional and organoleptic qualities, undesirable health effects of such treatments and expensive equipment required for other degradation techniques has encouraged recent emphasis on biological methods (Samarajeewa et al. 1990). Some microbial isolates have been reported with different levels of degradation abilities. The use of microorganisms offers for the control or elimination of aflatoxins in foodstuffs (Alberts et al .,2009) several lactic acid bacteria (LAB) strains have shown different capabilities for binding AFM1 in milk (Bovo et al .,2012; El-Nezami et al .,1998; Haskard et al .,2001; Kabak and Var, 2008; Pierides et al. ,2000). Lactic acid bacteria (LAB) and *bifidobacteria,* due in large part to their GRAS status and use as probiotics, are of particular interest for reducing the bioavailability of AFs. A number of studies have screened these microorganisms for the ability to bind to AFs and have reported a wide range of genus, species and strain specific binding capacities (Bolognani et al. 1997; El-Nezami et al. 1998b; Peltonen, 2000, 2001; Haskard et al. 2001; Lee et al. 2003; Hwang et al. 2005; Zinedine et al. 2005; Shahin, 2007).

MATERIALS AND METHODS

Materials

Bacterial and yeast strains

Kefir starter (cominox compani, spain) and *lactobacillus casei* JQ412732/1 (isolated from dairy products) which recorded in the National center for biotechnology information advances science and health (NCBI) were used in this study.

Contamination of milk and kefir production

2.4 mL AFM_1, standard solution (0.05 µg/ ml) was suspended again in 237.6 ml of low –fat sterilized milk (1.5 % fat), which was accidentally bought from a local supermarket in Kazerun-Iran, to a concentration of 500 pg of AFM, ml^{-1}. Kefir samples prepared from milk with AFM_1 (500 pg AFM_1/ml), as before explained. In order to evaluate the ability of kefir starter and *Lb. casei* ten tubes were pay attention which divided into two groups. There whit, two tubes were observed as control samples. First group involved five tubes each including 10 ml of contaminated milk were prepared. Different doses of kefir starter 0.4, 0.8, 1.2, 1.6, and 2gr (4, 8, 12, 16 and 20 %) were put

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to the tubes sequentially and mixed appropriately so that kefir starter was uniformly spread. Second group involving five tubes each including 10 ml of contaminated milk and fixed amount of kefir starter (0.8 g r which equals to 8%).*Lb. casei* was added directly to whole tubes in different concentrations: 0.01, 0.03, 0.05, 0.07 and 0.09 and mixed appropriately. Then, all the ten tubes were put in incubator at 24p C for 24h. Then coagulum was isolated from the milk by filtering with a filter paper and the liquid was spread in caps then put in incubator at 14p C for 24h. At last, kefir samples were kept at 4p C in refrigerator for 48 h, then ELISA test procedure was done. In order to prepare control samples two tubes (C_1 AND C_2) were observed, C_1 for assessing the initial contamination of milk which included 10ml of milk. It was kept at 4p C in refrigerator for 48 h, and at lasty ELISA test procedure was done. C_2 contained 10 ml of contaminated milk plus 0.1% (0.01 g) *Lb. casei* which was added immidiately and mixed appropriately. Then the tubes incubated at 38p C for 8h and at last kept at 4p C in refrigerator for 48h. Then, ELISA test procedure was done.

AFM_1 analysis

AFM_1 analysis was done by ELISA procedure as stated to Europroxima B.V. suggestion. The whole samples were centrifuged (at 2000 for 10 min at 4 c) and the supernatant liquids were analyzed for AFM_1 remains using direct competitive Enzyme-Linked Immunosorbent Assay (dc-ELISA) method. The ELISA system (Bio Tek, USA) included of ELISA reader (model ELx 808), ELISA washer (model ELx50) and the ELISA kit (Euro Proxima). In the direct competitive ELISA (de-ELISA)test, the 96- wells ELISA plate covered with anti-AFM_1 antibodies (clones G11, 6G4, and ATX2) was sued. 100 µL of the supernatant liquid was immidiately used per well. 100 µL of the AFM_1 standard solutions and test samples (100µl/ well) in repeatedly were put to the wells of microtiter plate and incubated for 1 hour at room temperature in the dark. The fluid was discarded the wells and the micro well holder was shaked upside down strongly (three times in a row) against absorbent paper to make sure complete removal of fluid from the wells. Whole the wells were made full with 250µl of rinsing buffer and cleared as described before. The washing process was repeated two time. 100 µL of the conjugate solution was added and incubated for 1 hour at room temperature (20-25pC) in the dark. The washing step was done three times. 100 µl substrate solution were added to each well and combined completely and incubated for 30 min at room temperature in dark. Then 100µL of the stop solution was put to each well, combined, and measured at wave long of 450 nm in ELISA reader.

Statistical analysis

Statistical analyses of AFM_1 removal tests were done by using the student's t-test for important differences between binding amounts of AFM_1 by the two microorganisms at different levels (kefir starter and *Lb. casei*). All process were performed in twice.

RESULTS AND DISCUSSION

Effect of kefir starter in detoxification of AFM_1

Figure 1 represents the result of kefie starter at various levels in AFM_1 decrease. AFM1 levels in kefir samples treated whit different doses of kefir starter ranged from 52.77% to 91.91% ., The highest decrease of AFM_1 was referred to the sample including 20% kefir starter (91.91%) and then

the samples containing 16 , 12 , 8 and 4% kefir starter had less quantities of decrease, respectively. The amounts of AFM_1 binding in these four samples were equal to 55.33, 55.53, 54.18and 52.77%. Therefore it is recommended to use 20% kefir starter to reach the highest reduction of AFM_1 in industrial production of kefir. There is no previous information on using kefir starter to remove kefir for AFM_1 (TABLE 1). The process implicated in kefir starter ability to bind aflatoxins remains unclear. It is currently believed that yeast cell wall has the ability to take the toxin.

Table 1. Effect of kefir starter in reduction of AFM1 in kefir

kefir	AFM_1 added to milk (pg ml^{-1})	Intitial AFM_1 in milk (pg ml^{-1})	reduced AFM_1 (pg ml^{-1})	reduced AFM_1 (%)
C_1	….	182		
T_1	500	682	359.918	52.77
T_2	500	682	369.524	54.18
T_3	500	682	378.75	55.53
T_4	500	682	377.393	55.33
T_5	500	682	626.871	91.91

Effect of *Lb.casei* in detoxification of AFM_1

Figure 2 represents the result of *Lb.casei* alone and in presence of kefie starter at various levels in AFM_1 decrease. After 48h, the results of our study showed that 0.1% *Lb.casei* (without kefir starter) removed 41.07% of AFM_1 content. AFM_1 levels in kefir samples dealt with various doses of *Lb.casei* and fixed amount of kefir starter (4%) ranged from 45.57-92.52% . , the sample including 0.9% *Lb.casei* whit 92.52% was more impressive in AFM_1 reduction and then the samples containing 0.7 , 0.5 , 0.3 and 0.1% starter had less quantities of reduction , respectively . The percentages of AFM_1 binding in these four samples were equal to45.57, 49.41, 62.99and90.18% respectively.

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Table 2. Effect of *L.casei* in reduction of AFM1 in kefir

L. casei	AFM_1 added to milk (pg ml^{-1})	Intitial AFM_1 in milk (pg ml^{-1})	reduced AFM_1(pg ml^{-1})	Reduced AFM_1 (%)
C2	500	682	280.1	41.07
T_6	500	682	308.777	45.57
T_7	500	682	337.01	49.41
T_8	500	682	429.652	62.99
T_9	500	682	615.03	90.18
T_{10}	500	682	631	92.52

Analysis of reduction of AFM_1 were carried out by HPLC method using *Lactobacillus acidifillus*, LAB pool and *S. cerevisiae* by Corrassin in 2013 The results of decrease aflatoxin M1 showed different amounts including 100%, 11.7± 4.4 and92 . 7±0. 7 respectively. . (Corrassin , 2013). And Co contaminated yogurt with 5 mg aflatoxin M_1 artificially and using HPLC method. They analized reduction of aflatoxin by different species of *Lactobacillus*. The results revealed that *Lactobacillus acidofillus Lf10, Streptococcus termophilus K45 , Lactobacillus bulgaricus* R21, *Lactobacillus helveticus* A34, *Lactobacillus rhamnosus GG* and *Lactobacillus rhamnosus LC705* reduced the amount of aflatoxin M1 to 18.4±0.5,28.2±4.3,31.4±2.6,29.4±1.5,48.4±2.8and49.6± 2.4respectively. (Motawee et al.2011). El khoury and Co assessed reduction of AFM_1 in yogurt using two LAB bacteriA including Bolgaricus and Termofilus in yogurt using ELISA method in 2011. in this test Aflatoxin was deleted to 87.6% by *Lactobacillus bulgaricus* and 70% by *Streptococcus termophilus*.In 2000 pierides and Co tested 5 species of LAB bacteria including, *Lactobacillus rhamnosus GG, Lactobacillus rhamnosus LC705, Lactobacillus gasseri, Lactobacillus acidophilus LA1* and *Lactobacillus rhamnosus stain 1/3* using HPLC method that showed reduction of AFM_1 ranged from 50/7% to 18/1% (El khoury et al.2011). Also Marhemati and Co carried out some researches on reduction of aflatoxin M_1 in Kefir by *L.Casei* bacteria using ELISA method that corresponded to our study. The results showed the reduction of aflatoxin to 69/19% by *L. Casei* also Kefir reduced AFM1 containing 85%. The maximum amount of AFM_1 absorbance by Kefir belonged to level 0.9. After that levels 0.7, 0.5, 0.3 and 1% showed less amounts of AFM1 reduction respectively (Marhamati et al.2014). So adding Kefir concentration increases AFM1 absorbance level. According to previous studies yeast cell wall has ability to absorb AFM1.

CONCLUSION

The results presented support the conclusions of previous researchers *Lactobacillus.casei* TD_4 and *saccharomyces kefir* strains, used in this study were shown effective in reducing the extent of AFM1 in kefir. Therefore, LAB seem to play a crucial role in AFM1 removal and could be used as a biological agent for AFM1 reduction.

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