Biomedical Research 2019; 30 (1): 122-126 *ISSN 0970-938X*
www.biomedres.info

Reduction of aflatoxin M1 by three acid- and bile-resistant antifungal probiotics *vs.* natamycin in milk.

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Abstract

Background and objectives: Aflatoxins (AFs) are produced by fungi, which may remain in the cow's milk even after pasteurization. Aflatoxin M1 (AFM1) is specifically of great medical importance, as it is certainly carcinogenic for human. Several strategies have been suggested for its reduction, including the use of probiotics, especially *Lactobacilli* or lactic acid bacteria (LAB). As this method has not been confirmed as a routine treatment, yet, in this study, we aimed to evaluate the effect of three LABs on reduction of AFM1 in traditional milk and cheese.

Materials and methods: In this study, 45 milk samples and 40 cheese samples were purchased from marketplaces of Shiraz city during February 2018-June 2018. Of 50 LABs purchased, the results of antifungal property, and resistance to bile salts, resulted in 5 strains. These 5 strains were tested for mean after addition of 5 ppm AFM1, compared to natamycin. The strains with reduction in AFM1 level were sequenced and registered in NCBI database.

Results: The results showed reduced AFM1 level in three LAB strains, sequenced as *Lactobacillus fermentum* CECT562 (T), *Lactobacillus brevis* ATCC14869 (T), *Enterococcus faecium* LMG 11423(T) to 0.05, 0.03, and 0.03, respectively.

Conclusion: The three LABs selected in the present study have significant effect on reducing AFM1 level in the traditional milk and cheese.

Keywords: Aflatoxins, Aflatoxin M1, *Lactobacillus*, Dairy products.

Accepted on January 23, 2019

Introduction

Milk serves as a main source of human nutrition for more than 10,000 years and a variety of dairy products are today produced from milk in different forms and flavors. Dairy intake has several health benefits for human, including the advantages to bone [1], cognitive health [2], and decreased incidence of diseases, like type 2 diabetes mellitus [3] and metabolic syndrome [4]. Nevertheless, numerous environmental contaminants, like pesticides, antibiotics, heavy metals and hormones, can enter cow's body, a part of which may remain not only in the raw milk, but also during collection and preparation process [5]. Although appropriate pasteurization, hygienic milk collection, and storage conditions can reduce the bacterial contamination of milk [6], they cannot eliminate the toxic contaminants, the most of important of which include mycotoxins [7].

Mycotoxins are small molecules produced by fungi as secondary metabolites that are harmful to humans, causing diseases and death [8]. Aflatoxins (AFs), produced mainly by *Aspergillus* (*A.*) *flavus* and *A. parasiticus*, are medically the most important mycotoxin, as they may damage the liver (hepatitis, edema, hemorrhagic necrosis) or cause liver, lung, and kidney carcinomas and immunosuppression [9]. The four main categories of AFs include B1 (AFB1), B2, G1, and G2. Ingestion of AFB1-contaminated feeds by the cow results in formation of the hydroxylated form, called aflatoxin M1 (AFM1), secreted in the cow's milk within 12 hours after the first ingestion. AFM1 is of great importance, as it is categorized as "certainly" carcinogenic to humans [10]. Several measures have been suggested to reduce the probability of this contamination, including the choice of hybrids, seeding time and density, lowest harvesting moisture and conservation temperature, suitable ploughing and fertigation and chemical or biological control [11];

nevertheless, AFM1 contamination of dairy products is still considered an important health hazard [12,13], especially in developing countries [14]. Iranian reports have defined high incidence of AFM1 contamination of cow milk with a high proportion exceeding the maximum tolerance limit accepted by European Union [15-17]; therefore, it is important to determine AFM1 levels in the different milks produced and take appropriate measures for its reduction.

Probiotics, defined as "live microorganisms that confer a health benefit on the host, when administered in adequate amounts" [18]. Probiotics, especially dairy strains of *Lactobacilli* or lactic acid bacteria (LAB), are suggested to reduce AFB1's toxicity in food [19] and dairy products, like yoghurt [20]. Hence, review studies suggest lack of sufficient evidence for global application of probiotics as an acceptable and efficient method for reducing AFs [21]. Due to the discrepancy in the results of studies in this regard, we aimed to evaluate the efficacy of three resistance *Lactobacilli* on reduction of AFM1 contamination of milk and cheese, compared to Natamycin.

Materials and Methods

Study design

The present study was approved by the Research Council of Shiraz University of medical sciences. In the first step, 45 samples of traditional milk and 40 samples of traditional cheese were collected from factories and marketplaces in Shiraz during February 2018-June 2018. All samples were sent to the laboratory immediately (while shaking) for culturing and the rest were kept in freezer until two months for any repetitions required. The presence of AFs and natamycin were detected by high performance liquid chromatography (HPLC); *Aspergillus* and *Penicillum* with ITS gene sequence and *Sacharomyces* and *Yarrowia* with D1/D2 gene sequence were separated according to the previously described method. Then, 50 LABs were purchased from Tak-Gene Company (Iran) and coded. For assessing the antifungal property of the LABs, the samples were cultured in methicillin-resistant *Staphylococcus aureus* (MRSA) media. AFM1 vials and natamycin powder were purchased from Farough Company, Iran. Then, the milk and cheese samples were assessed in 6 groups:

1. Group 1 (control 1): the samples of the traditional milk and cheese without fungi, AFM1, or natamycin, kept in dextrose chloramphenicol agar at 22-25°C for 5 days.

2. Group 2 (control 2): the samples of the traditional milk and cheese inoculated with AFM1 toxin, kept in dextrose chloramphenicol agar at 22-25°C for 5 days.

3. Group 3 (control 3): the samples of the traditional milk and cheese inoculated with Natamycin, kept in dextrose chloramphenicol agar at 22-25°C for 5 days.

4. Group 4 (case 1): 85 samples of traditional milk and cheese purchased from the marketplaces of Shiraz city were kept in dextrose chloramphenicol agar at 22-25°C for 5 days.

5. Group 5 (case 2): infected milk samples inoculated with the selected resistant LABs (8×10^3 cfu), measured by 0.5 McFarland method.

6. Group 6 (case 3): infected milk samples with natamycin (8×10^3 cfu).

The five LABs with bile resistance and antifungal property were coded as TD1/2, T21/2, T23/2, TD11, and LAX152, of which three were resistant to acid conditions. For comparison of the ability of these three in reducing the AFs, 0.5 ppm AF vial was added to 1000 cc traditional yoghurt and shaken well by shaker. After 120 minutes kept at 37°C, each 10 cc were kept in one tube and colonies were cultures in the tubes. After incubation at 30°C for 72 hours, the samples were sent to Farough Laboratory for measurement of AFM1 levels. The test was performed for all 5 samples of LABs. Also, the milk sample inoculated with 0.5 ppm or 0.2 g natamycin were tested for the level of AFM1.

In the final step, the strains of LABs reducing AFM1 were registered in NCBI database: https://submit.ncbi.nlm.nih.gov/

Statistical analysis

Results were presented as mean ± standard deviation (SD) for quantitative variables and by frequency (percentage) for qualitative variables. The mean level of AFM1 was compared between the groups using ANOVA and the pairwise comparison by Tukey test. Categorical variables were compared using chi-square test. For the statistical analysis, the statistical software IBM SPSS Statistics for Windows version 21.0 (IBM Corp. 2012. Armonk, NY: IBM Corp.) was used. P values of 0.05 or less were considered statistically significant.

Results

The results of which showed 28 strains could completely eliminated fungi in the media. For assessing the resistance of LABs to bile salts, 1%, 3%, and 5% bile salts were added to the MRSA media, the results of which showed only 5 of the 28 strains resistance to bile salts, coded as TD1/2, T21/2, T23/2, TD11, and LAX152. In the final step, for assessing the resistance of the 5 remaining LABs to acidic PH, they were tested in MRSA in acidic conditions for 120 minutes, the results of which revealed three strains with the property of resistance to acidic conditions: TD1/2, T23/2, and TD11. These three strains were selected as the final sample and referred for molecular test of PCR with rRNA S16.

The results of testing the six groups showed that the group without LABs, inoculated with AFM1 and natamycin showed no reduction in the level of AFM1 (0.5 ppm). Among the 5 groups with 5 strains of LABs, the mean level of AFM1 in the groups inoculated with TD1/2, TD21/2, TD23/2, TD11, and LAX152 were about 0.05, 0.03, 0.03, 0.01, and 0.05, respectively.

The three LABs with resistance to bile salts and acidic conditions, and antifungal property included TD1/2, TD 11,

and TD23/2 strains, for which the results of 16 s rRNA sequencing are shown in Tables 1 and 2.

Table 1. The results of 16 s rRNA sequencing for TD1/2, TD 11, and TD23/2 strains.

Code	Name	Number of nucleotides	Code of phylogenetic nomenclature	NCBI registration code
TD1/2	*Lactobacillus fermentum* CECT562(T)	1536	AJ575812	MH685411
TD11	*Lactobacillus brevis* ATCC14869(T)	1527	K1271266	MH685412
TD23/2	*Enterococcus faecium* LMG 11423(T)	1536	AJ301830	MH685413

Table 2. Genetic record of NCBI of Lactobacillus bacteria.

Code	Name	Search site
TD1/2	*Lactobacillus fermentum* CECT562(T)	https://submit.ncbi.nlm.nih.gov/SUB4342367
TD11	*Lactobacillus brevis* ATCC14869(T)	https://submit.ncbi.nlm.nih.gov/SUB4342371
TD23/2	*Enterococcus faecium* LMG 11423(T)	https://submit.ncbi.nlm.nih.gov/SUB4342375

Discussion

The results of testing 50 LABs indicated that only 5 had both antifungal activity and resistance to bile salts. Addition of 0.5 ppm AFM1 to these 5 strains showed significant reduction in mean AFM1 level. The three LABs with resistance to bile salts and acid PH were sequenced, the results of which revealed them as *Lactobacillus fermentum*, *Lactobacillus brevis*, and *Enterococcus faecium* strains. These results confirm the effect of these three LABs on reduction of AFM1 in milk and cheese and suggest their application as bio preservatives.

Several Iranian reports have indicated a high level of AFs in dairy products [15-17], which indicate the necessity to pay greater attention to the strategies reducing AFs in dairy products of Iran, where there is appropriate ecologic condition for producing dairy products and different forms of dairy products are routinely used. Of note, dairy products are produced and sold in Iran in two forms of traditional and industrial. While industrial products can be found in every supermarket, several customers prefer the traditional dairy products, for their higher chance of natural benefits, and less factory processing; meanwhile, the lower quality control on these products may result in higher infection of these products to several bacteria and fungi [22,23].

According to the significance of fungal infection and AFs produced for human health [10,11], different studies have evaluated the presence of LABs in different dairy products for reducing fungal contamination of dairy products. As the results of the present study indicated, 28 of 50 LABs had antifungal activity and only 3 of the total LABs investigated were highly potent for reducing AFM1. In the study by Prabhurajeshwar et al. of 30 LABs examined, isolated from curd, only 16 were resistant to bile salts and acids [24]. These results confirm that of the present study on the fact that not all LABs have antifungal property and their properties should be examined before use. Other studies have isolated LABs from different dairy products and tested their ability of reducing AFs [20,25,26]. The study by Sadeghi et al. isolated *Lactobacillus acidophilus* and brevis from traditional sourdough and tested them on the growth of *Aspergillus flavus* and reduction of AFB1; they reported significant reduction in the level of AFB1 and thus suggested these two LABs (especially non-viable cells) as efficient bio preservatives for dairy products [27]. The study by Verma et al. also reported that of 18 Bacillus species, *Lactobacillus brevis* was the most sensitive for reduction of AFM1 in milk [28]. The results of the two studies [27,28] confirm that of the present study on the effect of *Lactobacillus brevis* on AF. Another study by Fazeli et al. isolated different LABs from sourdough and reported significant reduction of AFB1 by three strains, including *Lactobacillus casei*, *plantarum*, and *fermentum* [29]. The results of this study confirm that of the present study on the effect of *Lactobacillus fermentum* on AF. Also, Nazhand et al. studied 20 LABs and isolated three with the ability to eliminate Coumarin (similar to AFs), among which two strains of *Enterococcus faecium* had the highest ability [30]. These results confirm that of the present study on the satisfactory effects of *Enterococcus faecium*. Although all the above-mentioned studies confirm the results of the present study on the effect of these three LABs on reducing AFs, the LAB strains and AF types investigated differed among studies and the three strains suggested in the present study has been introduced here for the first time.

The beneficiary effects of LABs, isolated from other foods, rather than dairy products, have also been confirmed; Farzaneh et al. isolated LABs form pistachio nuts and reported apparent decreases in AFB1 levels after 24 hours in in cell free supernatant at 35-40°C. Shokryazdan et al. isolated 140 LABs from human milk, infant feces, and fermented grapes and dates and described them as efficient antimicrobial probiotic strains [31]. Therefore, different LABs can be found in different foods and many have antifungal property and can be used for reducing the AFB1 levels after appropriate tests. In the present study, three LABs were introduced with significant effect of reducing AFM1 levels in milk and cheese.

As to the evidence, various factors may play a role in the efficiency of LAB on reduction of AFs and fungal growth, including the strain of the bacteria. Different bacteria may have various mechanisms for removal of AFs, such as binding to the fungal membrane (for which the cell wall peptidoglycans and polysaccharides of the bacteria are important) and inhibiting absorption of amino acids; this difference causes dissimilar antifungal potencies for various LABs [32]. In the present study, we examined the strains with antifungal property and resistance to bile salts and acids for their applicability in milk

and cheese and the results showed that only three of the 50 LABs tested had all the three properties. Furthermore, these three LABs had different potencies for reducing AFM1 levels and *Lactobacillus* brevis and fermentum had the greatest reduction of AFM1 levels. The incubation period and temperature are also suggested important factors for inhibiting the fungal growth. Although the best incubation period and temperature was suggested at about 48 hours and 25-30°C [33], different studies have used different incubation periods and temperatures, for instance Fazeli et al. incubated LABs in the presence of AFB1 at 37°C for a period of 72 hours and reported that the percent of AFB1 removal of the strains differed according to different incubation periods and a higher AFB1 removal was observed in 72 hour *vs.* 24 hour old cultures [29]. In the current study, we incubated the samples at 22-25°C for 5 days and observed sufficient results. Furthermore, different inoculum dose of treatment have been reported as sufficient bacterial population for elimination of AFs in different studies [25,26]. In the study by Sadeghi et al., the required bacterial population for *Lactobacillus* brevis was 2×10^3 cfu [27]. Fazeli et al. also reported 2×10^3 cfu as the sufficient amount for removal of AFs in *Lactobacillus casei*, *plantarum*, and *fermentum* [29]. In the present study, a mean value of 8×10^3 cfu: 3×10^3 cfu for *Lactobacillus* fermentum and 5×10^3 cfu for *Enterococcus faecium* and the results showed sufficient inoculum dose of treatment for these strains.

The present study could successfully isolate the LABs with antifungal property and resistance to bile salts and acid, compared to natamycine. Nevertheless, this study could have some limitations, such as the.

Conclusion

In conclusion, the results of this study showed that among 50 LABs, only 28 had antifungal properties. For their applicability in human body, we tested their resistance to bile salts and acids and the results showed that only three LABs had all the three desired characteristics. We then tested their ability to reduce AFM1 in comparison with natamycin and the two LABs sequenced as *Lactobacillus* brevis, fermentum and Enterococcus faecium strains had the greatest ability to reduce AFM1; we finally registered the details of the three strains in NCBI database. Thus, we suggest addition of these three strains to the traditional milk and cheese of Shiraz city, which showed to have a high fungal contamination.

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