

Original Article

Identification of Dairy Fungal Contamination and Reduction of Aflatoxin M₁ Amount by Three Acid and Bile Resistant Probiotic Bacteria

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Abstract

Aflatoxins (AFs) released by fungi are observed in the cow's milk even after pasteurization. Aflatoxin M₁ (AFM₁) has particularly an incredible clinical significance, as a critical carcinogenic agent for humans. Several strategies have been implemented for lowering the AFM₁ amount, such as the employment of probiotics, particularly lactobacilli or lactic acid bacteria (LAB). However, this strategy has not been applied routinely until today. This study aimed to evaluate the effect of three LABs on the reduction of AFM₁ in traditional milk and cheese samples. In total, 85 milk (n=45) and cheese (n=40) samples were obtained from the open markets of Shiraz, Iran, from February to June 2018. Additionally, the AFM₁ levels were evaluated, compared to those of the *National Iranian Standard*. The data were then analyzed in SPSS software (version 20) through the Chi-square test. Statistical analysis was performed at a 95% confidence level (p-value of <0.00001). Out of 50 purchased LABs, the efficient antifungal property and resistance to bile salts were observed in five strains. The mean value of these five strains was calculated after adding 5 ppm AFM₁, compared to natamycin. The strains with a reduction in AFM₁ level were sequenced and registered in the NCBI database. In total, 15 samples with contamination higher than the allowed limit included *Penicillium* spp, *Aspergillus niger*, *Saccharomyces cerevisia*, *Saccharomyces paradoxus*, and *Yarrowia lipolytica*. The results also showed reduced AFM₁ levels in three LAB-treated strains. *Lactobacillus fermentum* CECT562 (T), *Lactobacillus brevis* ATCC14869 (T), and *Enterococcus faecium* LMG 11423 (T) had this capability to 0.05, 0.03, and 0.03 respectively. The *National Iranian Standard* should be implemented to have control over traditional dairy products with more care. The three LABs selected in the current study revealed a significant effect on reducing AFM₁ levels in traditional milk and cheese.

Keywords: Aflatoxin M₁, Contamination, *Lactobacillus*, Probiotics

Identification de la Contamination Fongique des Produits Laitiers et Réduction de la Quantité D'aflatoxine M₁ par Trois Bactéries Probiotiques Résistantes aux Acides et à la Bile

Résumé: les aflatoxines (AFs) libérées par les champignons sont observées dans le lait de vache même après la pasteurisation. L'aflatoxine M₁ (AFM₁) a une signification clinique particulièrement incroyable, en tant qu'agent cancérigène critique pour l'homme. Plusieurs stratégies ont été mises en œuvre pour réduire la quantité d'AFM₁, telles que l'utilisation de probiotiques, en particulier les lactobacilles ou les bactéries lactiques (LABs). Cependant, cette stratégie n'a pas été appliquée en routine jusqu'à aujourd'hui. Cette étude visait à évaluer l'effet

de trois LABs sur la réduction de l'AFM₁ dans les échantillons de lait et de fromage traditionnels. Au total, 85 échantillons de lait (n = 45) et de fromage (n = 40) ont été obtenus sur les marchés ouverts de Shiraz, en Iran, de février à juin 2018. De plus, les niveaux d'AFM₁ ont été évalués, comparés à ceux de la *norme nationale iranienne*. Les données ont ensuite été analysées dans le logiciel SPSS (version 20) grâce au test du chi carré. L'analyse statistique a été effectuée à un niveau de confiance de 95% (valeur-p <0.00001). Sur 50 LABs achetés, la propriété antifongique efficace et la résistance aux sels biliaires ont été observées dans cinq souches. La moyenne de ces cinq souches calculée après addition de 5 ppm d'AFM₁, par rapport à la Natamycine. Les souches avec une réduction du taux d'AFM₁ ont été séquencées et enregistrées dans la base de données NCBI. Au total, 15 échantillons avec une contamination supérieure à la limite autorisée comprenaient *Penicillium spp*, *Aspergillus niger*, *Saccharomyces cerevisia*, *Saccharomyces paradoxus* et *Yarrowia lipolytica*. Les résultats ont également montré des niveaux réduits d'AFM₁ dans trois souches traitées au LAB. *Lactobacillus fermentum* CECT562 (T), *Lactobacillus brevis* ATCC14869 (T) et *Enterococcus faecium* LMG 11423 (T) avaient cette capacité à 0.05, 0.03 et 0.03 respectivement. La *norme nationale iranienne* devrait être mise en œuvre pour avoir un contrôle sur les produits laitiers traditionnels avec plus de soin. Les trois LABs sélectionnés dans l'étude actuelle ont révélé un effet significatif sur la réduction des niveaux d'AFM₁ dans le lait et le fromage traditionnels.

Mots-clés: Aflatoxine M₁, Contamination, *Lactobacillus*, Probiotiques

Introduction

Factors of interest in fungal contamination of dairy products include the ability to grow at low temperatures, fermentation of sucrose and lactose, production of hydrolyzing enzymes for fatty and protein hydrolysis of the product, production of lactic acid and citric acid in the product, and resistance against chemical holders (Caroli et al., 2011). It is notable that probiotics play a paramount role in human health (Elwood et al., 2007). Cow' milk undergoes a variety of contaminants, which threaten human health (Khaniki, 2007). Although accurate pasteurization, safe milk collection, and storage conditions alleviate the microbial contamination to a high level, they cannot alleviate toxic contaminants, particularly mycotoxins that need more process to be eliminated (Girma et al., 2014).

Mycotoxin small compounds are produced by fungal agents as secondary metabolites that lead to serious disorders and even human death. Aflatoxins (AFs), mainly produced by *Aspergillus flavus* (*A. flavus*) and *A. parasiticus*, as superintendent agents, damage the liver (hepatitis, edema, and hemorrhagic necrosis) or lead to liver, lung, and kidney cancers, as well as immunosuppression (Lizárraga-Paulín et al., 2011).

The four major AFs categories include B₁ (AFB₁), B₂, G₁, and G₂. AFB₁-containing nutrients by the cow culminate in the formation of the hydroxylated form, known as aflatoxin M₁ (AFM₁), released from the cow's milk within 12 h after the first consumption.

AFM₁ is regarded as a critical carcinogenic compound to humans (Dhanasekaran et al., 2011). Several strategies have been proposed toward the reduction of the levels of this compound in dairy products, including seeding time and density, chemical control, choice of hybrids, extreme lowering of grass moisture and keeping temperature, proper plowing and fertigation, and application of biological routes.

However, dairy products contamination with AFM₁ toxin remains a highly considerable health problem (Pena-Rodas et al., 2018), particularly among developing countries. Noticeably, the high incidence of AFM₁ contamination in cow milk at higher levels, compared to the maximum tolerance limit approved by the European Union is present in Iran (Ghazani, 2009). Therefore, it is of critical importance to determine the AFM₁ levels in various milk products and accurate measures for its reduction.

Probiotics, considered as "live microorganisms", exert paramount health advantages in favor of existing host when administered in adequate amounts" (Hill et

al., 2014). Of particular, dairy strains of *Lactobacilli* or lactic acid bacteria (LAB) have been introduced to reduce AFB₁'s amount in food and dairy products, including yogurt. Accordingly, review studies have revealed the lack of sufficient document data regarding the vast and common application of probiotics as an approved and proper approach toward reducing AFs (Adebo et al., 2017). Owing to the uncertainty in the findings of the studies in this regard, this study aimed to evaluate the milk and cheese contamination level and applicability of three resistance *Lactobacilli* on the reduction of AFM₁ levels, compared to Natamycin.

Material and Methods

Samples and Fungal Agents. The study protocol was approved by the Research Council of Shiraz University of Medical Sciences, Shiraz, Iran. In total, 85 samples of traditional milk (n=45) and cheese (n=40) were obtained from factories and open markets in Shiraz, Iras, from February to June 2018. All samples were transferred to the laboratory promptly (while shaking) for culture, and the remained were kept in the freezer for two months for further investigations.

Fungal contamination was investigated with phenotypic tests. Moreover, the PCR technique was used for the identification of yeasts and mold for molecular confirmation of strains belonging to one type and genotyping. Obtained sequences were analyzed at the online database of *Gene Bank NCBI*. In such cases, sequence identification of genes in the areas of S rRNA and ITS was performed by the primers ITS1 and ITS4, as well as NS1 and NS8, respectively. Therefore, molecular identification of yeasts was initiated by sequence detection of gene areas D1/D2 in *IBC*. Dairy samples were used for the purification and sequencing of isolated yeasts and mold colonies. To ensure, the samples were sent to *IBC*, and separated colonies were identified and registered in *Gene Bank, NCBI*.

AFs-levels Assessment. The existence of AFM₁ and natamycin was clarified using High-Performance Liquid Chromatography. Afterward, *Aspergillus* and *Penicillium*, as well as *Saccharomyces* and *Yarrowia*

with ITS and D1/D2 gene sequences, respectively, were isolated following a predefined method. Subsequently, 50 LABs were purchased from Tak-Gene Company (Iran) and coded. Regarding the evaluation of the antifungal traits 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. In the next stage, the milk and cheese samples were ordered in six groups as follows:

Group 1 (control 1): The traditional milk sample without fungi, AFM₁, or natamycin was kept in Sabouraud dextrose chloramphenicol agar at 22-25°C for 5 days.

Group 2 (control 2): The traditional milk sample inoculated with fungi and AFM₁ toxin was kept in Sabouraud dextrose chloramphenicol agar at 22-25°C for 5 days.

Group 3 (control 3): The traditional milk sample inoculated with Natamycin was kept in Sabouraud dextrose chloramphenicol agar at 22-25°C for 5 days.

Group 4 (case 1): Traditional milk samples (n=85) purchased from the marketplaces of Shiraz, Iran, were kept in Sabouraud dextrose chloramphenicol agar at 22-25°C for 5 days.

Group 5 (case 2): Infected milk samples inoculated with the selected resistant LABs (8×10³ CFU) with turbidity equal to 0.5 McFarland standard value.

Group 6 (case 3): Infected milk samples with natamycin (8×10³ CFU).

To assess the resistance of LABs to bile salts, 1%, 3%, and 5% bile salts were added to the MRSA media. Out of five bile-tolerant LABs containing antifungal features, five LABs were resistant to acid conditions and coded as TD1/2, T21/2, T23/2, TD11, and LAX152. Considering the comparison of the AFs-reducing ability of these three strains, 0.5 ppm of AF vial was inoculated into 1000 mL traditional yogurt and shaken completely by a shaker and left at 37°C for 120 min. Afterward, each 10 mL was dropped into one tube, and the colonies were cultured in them. After incubation at 30°C for 72 h, the samples were

transported to a laboratory for AFM₁ level evaluation. The test was performed for all five LABs. Furthermore, the milk samples inoculated with 0.5 ppm or 0.2gr natamycin were tested for the level of AFM₁. In the final step, the AFM₁-reducing LABs strains were registered in the NCBI database: <https://submit.ncbi.nlm.nih.gov/>

Statistical Analysis. The data were analyzed in IBM SPSS software (version 21.0) (IBM Corp. 2012. Armonk, NY: IBM Corp.) The quantitative and qualitative variables were presented as mean±SD and frequency, respectively. Moreover, the groups were compared in terms of the mean level of AFM₁ using ANOVA, and the pairwise comparison was performed by Tukey's test. Categorical variables were also compared using the Chi-square test. A p-value less than 0.05 was considered statistically significant.

Results

This study evaluated 85 samples of dairy products, including cheese (n=40; 47.66%) and traditional milk samples (n=45; 94.52%). Table 1 tabulates the analysis results of the presence of aflatoxin in the samples taken. Of the total samples, 39 (9.45%) and 31 (36.4%) ones were desirable and acceptable, respectively. Moreover, the number of mold colonies and colonies was more than the acceptable limit and considered unacceptable in 15 cases (17.6%). Therefore, 17.6% of the samples had mold and yeast contamination. Furthermore, the results of the test showed no significant difference between the amount of mold and

yeast infection (P=0.05).

The dairy product samples were collected from Shiraz (n=85), and among these isolates (i.e., *Penicillium* strains, *Aspergillus niger*, *Saccharomyces cerevisia*, *Saccharomyces paradoxus*, and *Yarrowia lipolytica*), the two molds, including *Aspergillus Niger* and *Penicillium* spp., had the ability of aflatoxin-bearing.

AFs-reducing traits of LABs

The results revealed that 28 strains could eliminate fungi in the media. Notably, only five out of 28 strains tolerated the bile salts, coded as TD1/2, T21/2, T23/2, TD11, and LAX152. In the final step, considering the assessment of the resistance of the five LABs to acidic conditions, they were kept in MRSA media in acidic pH for 120 min, the results of which revealed three strains with the property of resistance to acidic conditions (i.e., TD1/2, T23/2, and TD11). These three strains were selected as the final sample and referred for the molecular test of PCR with 16srRNA.

The results of analyzing the six groups showed that the group without LABs inoculated with AFM₁, and natamycin indicated no reduction in the level of AFM₁ (0.5 ppm). Among the five groups with five strains of LABs, the mean levels 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 the bile salts and acidic conditions and antifungal property included TD1/2, TD 11, and TD23/2 strains, for which the results of 16s rRNA sequencing are shown in Table 2.

Table 1. Analysis of the presence of aflatoxin in the samples

Production dose level	Aflatoxin produced ppb	Sample	Sample code
<0.03ppb	+	Cheese	01
<0.03ppb	+	Milk	02
ND	+	Cheese	03
0/023ppb	+	Cheese	04
ND	-	Cheese	05
ND	-	Milk	06
0.03ppb	+	Milk	07
0.03ppb <	+	Milk	08
0.02ppb	+	Cheese	09
-	-	Milk	010
-	-	Milk	011
-	-	Cheese	012

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

Code	Name	Number of nucleotides	Code of phylogenetic nomenclature	of NCBI registration code	Search site of NCBI
TD1/2	<i>Lactobacillus fermentum</i> CECT562(T)	1536	AJ575812	MH685411	https://submit.ncbi.nlm.nih.gov/SUB4342367
TD11	<i>Lactobacillus brevis</i> ATCC14869(T)	1527	K1271266	MH685412	https://submit.ncbi.nlm.nih.gov/SUB4342371
TD23/2	<i>Enterococcus faecium</i> LMG 11423(T)	1536	AJ301830	MH685413	https://submit.ncbi.nlm.nih.gov/SUB4342375

Discussion

In this study, the *Penicillium* genus was the common dairy-contaminating genotype, followed by *Aspergillus niger*, *Saccharomyces cerevisia*, *Saccharomyces paradoxus*, and *Yarrowia lipolytica*. Out of 85 dairy product samples collected from Shiraz, the molds were isolated from *Aspergillus niger* and *Penicillium* strains. It should be mentioned that most of the isolates had the ability of aflatoxin-bearing.

Out of 50 LABs, only five exhibited antifungal activity and bile salt resistance traits. Inoculation of 0.5 ppm AFM₁ to them was associated with significant amelioration in mean AFM₁ level. These three LABs were sequenced and uncovered as *L. fermentum*, *L. brevis*, and *E. faecium* strains existed.

Several surveys have indicated high levels of AFs in dairy products (Oveisi et al., 2007; Ghazani, 2009; Hashemi, 2016), necessitating higher attention to the AFs reducing approaches in dairy products. Notably, dairy products are produced and sold in two traditional and industrial forms in the country. Although industrial products are wider, some others prefer traditional dairy products (Tajkarimi et al., 2008; Momtaz et al., 2012).

According to the significance of fungal infection and AFs produced for human health (Prandini et al., 2009; Dhanasekaran et al., 2011), various studies have

evaluated the presence of LABs in dairy products for reducing their fungal contamination. It was observed that 28 out of 50 LABs exerted antifungal activity and three of them were highly potent for reducing AFM₁. In a previously conducted study, 16/30 of LABs isolated from the curd tolerated bile salts and acids (Prabhurajeshwar and Chandrakanth, 2017), highlighting our findings that not all LABs exert antifungal traits requiring prior evaluation. The LABs from a variety of dairy products have shown the ability to reduce AFs (Abdelmotilib et al.; Hernandez-Mendoza et al., 2010; Elsanhoty et al., 2014).

Sadeghi et al. (2016) isolated *L. acidophilus* and *L. brevis* from traditional sourdough and examined inhibitory growth on *A. flavus* and reduction of AFB₁. The results showed a significant reduction in the AFB₁ level; accordingly, these LABs (especially non-viable cells) were supposed as proper bioremediation for dairy products. In another study, *L. brevis* was the most sensitive agent to reduce the AFM₁ level in milk (Verma et al., 2013).

The findings of these studies (Verma et al., 2013; Sadeghi et al., 2016) highlight our results regarding the effect of *L. brevis* on AF levels. Fazeli et al. (2009) isolated various LABs from sourdough and found a significant reduction of AFB₁ by *L. casei*, *L.*

plantarum, and *L. fermentum*. The results of this study highlighted the inhibitory effect of *L. fermentum* on AFs levels. Moreover, Nazhand et al. (2017) studied 20 LABs from which two strains of *E. faecium* had the highest ability to eliminate Coumarin (similar to AFs). These findings are in line with the results of the present study on the satisfactory effects of *E. faecium*. Although all the above-mentioned studies highlighted our observations on the AFs reducing effect of these three LABs, the investigated LAB strains and AF types varied among studies, and the three strains in the present study have been introduced here for the first time.

Various agents determine the efficiency of LAB in AFs reduction and fungal development, such as the bacterial strain. Various bacteria employ different mechanisms for the removal of AFs, such as binding to the fungal membrane (for which the cell wall peptidoglycans and polysaccharides of the bacteria are important) and preventing the absorption of amino acids leading to dissimilar antifungal potencies for various LABs (Perczak et al., 2018).

The strains were evaluated in this study with antifungal property and resistance to bile salts and acids for their applicability in milk and cheese, and only three of the 50 LABs had all three properties. Furthermore, these three LABs had different potencies for reducing AFM₁ levels; moreover, *L. brevis* and *L. fermentum* had the highest reduction of AFM₁ levels.

However, the best incubation period and the temperature were supposed about 48 h at 25-30°C (Dalié et al., 2010). A variety of incubation periods and temperatures have been used in this regard. Fazeli et al. (2009) incubated LABs in the existence of AFB₁ at 37°C for 72 h and observed a difference between the 72- and 24-h cultures in terms of AFB₁ removal by the strains.

Noticeably, our samples were incubated at 22-25°C for five days, and sufficient results were observed in this regard. Furthermore, different inoculum amount of treatment has been reported as sufficient bacterial population for the elimination of AFs in different

studies (Abdelmotilib et al.; Hernandez-Mendoza et al., 2010). In a study conducted by Sadeghi et al. (2016), the required bacterial population for *L. brevis* was 2×10^3 CFU. Fazeli et al. (2009) also reported 2×10^3 CFU as a sufficient amount for the removal of AFs in *L. casei*, *L. plantarum*, and *L. fermentum*.

In the present study, regarding the 8×10^3 CFU, the mean values of 3×10^3 and 5×10^3 CFU were obtained for *L. fermentum* and *E. faecium*, respectively. Moreover, the results showed a sufficient inoculum dose of treatment for these strains. This survey could successfully isolate the LABs with antifungal traits and resistance to bile salts and acidic conditions, compared to natamycin.

Conclusion

It was observed that out of 50 LABs, 28 ones contained antifungal properties. Regarding their applicability in human body conditions, their resistance to bile salts and acids was tested, and the results outlined that only three LABs had all the beneficial characteristics. The ability to reduce AFM₁, compared to natamycin, revealed that the LABs sequenced as *L. brevis*, *L. fermentum*, and *E. faecium* strains had the highest ability to reduce AFM₁. The details of the three strains were registered in the NCBI database. Accordingly, it is suggested to employ these three strains to the traditional studied milk and cheese samples, which had a high level of fungal contamination.

Authors' Contribution

Study concept and design: F. F. Sh.

Acquisition of data: F. F. Sh.

Analysis and interpretation of data: M. T. E. and M. B.

Drafting of the manuscript: F. F. Sh. and V. R.

Critical revision of the manuscript for important intellectual content: M. T. E. and M. B.

Statistical analysis: F. F. Sh. and J. H.

Administrative, technical, and material support: F. F. Sh. and M. T. E.

Ethics

All samples were collected using aseptic methods and in conformity with the ethical guidelines of the 1975 declaration of Helsinki.

Conflict of Interest

The authors declare that they have no conflict of interest.

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