Identification dairy fungal contamination and reduction of aflatoxin M₁ amount by three acid– and bile–resistant probiotic bacteria

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Abstract

Background: Aflatoxins (AFs) released by fungi, withstand in the cow’s milk even after pasteurization. Aflatoxin M₁ (AFM₁) has particularly an incredible clinical significance, as a critical carcinogenic agent for human. Several strategies have been implemented for lowering AFM₁ amount, such as the employment of probiotics, particularly those Lactobacilli or lactic acid bacteria (LAB). Nevertheless, this strategy not been applied routinely until today.

Objectives: In this study, we designed to evaluate the effect of three LABs on the reduction of AFM₁ in traditional milk and cheese.

Materials and Methods: In this study, 45 milk samples and 40 cheese samples were obtained from the open market of Shiraz city in the course of Feb–June 2018. Additionally, the AFM₁ levels were compared to the National Iranian Standard and were analyzed by SPSS version 20 and chi-square statistical analysis with about 95% accuracy and significant level (p-value<0.00001). Of 50 LABs purchased, the efficient antifungal property, and resistance to bile salts was observed in 5 strains. These 5 strains were tested for mean afterward adding of 5 ppm AFM₁, compared to natamycin. The strains with reduction in AFM₁ level were sequenced and registered in NCBI database.

Results: Fifteen samples had contamination higher than allowed limit having Penicillium spp, Aspergillus niger, Saccharomyces cerevisia, Saccharomyces paradoxus, and Yarrowia lipolytica.
The results outlined reduced AFM$_1$ level in three LAB-treated strains. *Lactobacillus fermentum* CECT562 (T), *Lactobacillus brevis* ATCC14869 (T), *Enterococcus faecium* LMG 11423(T) had this capability to 0.05, 0.03, and 0.03 respectively.

**Discussion:** The *National Iranian Standard* obedience must be performed and controlled with more care in traditional dairy products. The three LABs selected in the current study revealed an important influence on reducing AFM$_1$ level in traditional milk and cheese.

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

**Introduction**

Factors of interest in fungal contamination of dairy products include the ability to grow at low temperatures, fermentation of sucrose and lactose, produce 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). Despite accurate pasteurization, safe milk collection, and keeping conditions alleviate the microbial contamination to a high level, the toxic contaminants; particularly mycotoxins need more process to be eliminated (Girma et al., 2014).

Mycotoxin small compounds are produced by fungal agents as secondary metabolites and cause serious disorders and even human death. Aflatoxins (AFs), mainly produced by *Aspergillus flavus* (*A. flavus*) and *A. parasiticus*, as superintendent agents, damaging the liver (hepatitis, edema, hemorrhagic necrosis) or lead to cancer of liver, lung, and kidney and immunosuppression (Lizárraga-Paulín et al., 2011). The four major AFs categories include B$_1$ (AFB$_1$), B$_2$, G$_1$, and G$_2$. Consumption of AFB$_1$–containing nutrients by the cow culminate in the formation of the hydroxylated form, known as aflatoxin M$_1$ (AFM$_1$), released from the cow’s milk within 12 hrs after the first consumption. AFM$_1$ is critical carcinogenic compound to human (Dhanasekaran et al., 2011). Several strategies have been proposed toward reduction of the levels of this compound in dairy products, including seeding time and density, chemical control, the choice of hybrids, extreme lowering of grass moisture and keeping temperature, proper ploughing and fertigation, and application of biological routes. However, dairy products contamination with AFM$_1$ toxin
remains a highly considerable health problem (Pena-Rodas et al., 2018); particularly among developing countries. Noticeably, this issue of high incidence of AFM$_1$ contamination in cow milk higher level than the maximum tolerance limit approved by European Union is present in Iran (Ghazani, 2009). Hence, AFM$_1$ levels determination in the various milk products and also accurate measures for its reduction is pivotal.

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 AFB$_1$’s amount in food and dairy products, including yoghurt. Thereby, review studies have revealed the lack of sufficient document data regarding vast and common application of probiotics as an approved and proper approach toward reducing AFs (Adebo et al., 2017). Owing to the uncertainty in findings of studies in this regard, this study aimed to evaluate the milk and cheese contamination level and applicability of three resistance Lactobacilli on reduction of AFM$_1$ levels compared to Natamycin.

Materials and Methods

Samples and fungal agents

The present study was approved by the Research Council of Shiraz University of medical sciences. Forty-five traditional milk and 40 traditional cheese samples were obtained from manufacturing works and open market in Shiraz during Feb–June 2018. All samples were transferred to the laboratory promptly (while shaking) for culture and the remained were kept in the freezer until two months for further investigations required.

Fungal contamination was investigated with phenotypic tests. Also, the PCR technique was used for identification of yeasts and mold for molecular confirmation of strains belonging to one type and genotyping. Obtained sequences were analyzed at 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. Hence, molecular identification of yeasts was initiated by sequence detection of gene areas D1 /D2 in IBC. Dairy samples were used for 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 by High Performance Liquid Chromatography (HPLC); Aspergillus and Penicillium with ITS gene sequence and Saccharomyces and Yarrowia with D1/D2 gene sequence were isolated as per the formerly defined method. Then, 50 LABs were purchased from Tak–Gene Company (Iran) and coded. For 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. Next, the milk and cheese samples were ordered in 6 groups:

1. Group 1 (control 1): the traditional milk sample without fungi, AFM1, or natamycin, kept in Sabouraud dextrose chloramphenicol agar at 22–25°C for 5 days.
2. Group 2 (control 2): the traditional milk sample inoculated with fungi, AFM1 toxin, kept in Sabouraud dextrose chloramphenicol agar at 22–25°C for 5 days.
3. Group 3 (control 3): the traditional milk sample inoculated with Natamycin, kept in in Sabouraud dextrose chloramphenicol agar at 22–25°C for 5 days.
4. Group 4 (case 1): 85 traditional milk sample purchased from the marketplaces of Shiraz city were kept in Sabouraud 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), with turbidity equal to 0.5 McFarland standard value
6. Group 6 (case 3): infected milk samples with natamycin (8×10^3 cfu). For assessing the resistance of LABs to bile salts, 1%, 3%, and 5% bile salts were added to the MRSA media.

The five bile-tolerant LABs containing antifungal features of which three were resistant to acid conditions, were coded as TD1/2, T21/2, T23/2, TD11, and LAX152. For comparison of the AFs-reducing ability of these three strains, 0.5 ppm of AF vial was inoculated into 1000 mL traditional yoghurt and shaken completely by shaker and left at 37°C for 120 min. Afterward, each 10 mL was dropped into one tube and colonies were cultured in them. After incubation at 30°C for 72 hrs, the samples were transported to a Laboratory for AFM1 levels 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 NCBI database: https://submit.ncbi.nlm.nih.gov/
**Statistical analysis**

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

**Results**

In this study, 85 samples of dairy products including 40 cheese samples (47.66%), 45 samples of traditional milk (94.52%) were studied. The results of an analysis of the presence of aflatoxin in the samples taken in Table 1 are presented. Of the total samples, 39 cases (9.45%) were desirable, 31 (36.4%) were acceptable and in 15 cases (17.6%) the number of mold colonies and colonies was more than the acceptable limit and was considered unacceptable. Thus, 17.6% of the samples had mold and yeast contamination. Also, the results of the test showed no significant difference between the amount of mold and yeast infection (P-value 0.05).

<table>
<thead>
<tr>
<th>Production dose level</th>
<th>Aflatoxin produced ppb</th>
<th>sample</th>
<th>Sample code</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.03ppb</td>
<td>+</td>
<td>cheese</td>
<td>01</td>
</tr>
<tr>
<td>&lt;0.03ppb</td>
<td>+</td>
<td>milk</td>
<td>02</td>
</tr>
<tr>
<td>ND</td>
<td>+</td>
<td>cheese</td>
<td>03</td>
</tr>
<tr>
<td>0.023ppb</td>
<td>+</td>
<td>cheese</td>
<td>04</td>
</tr>
<tr>
<td>ND</td>
<td>-</td>
<td>cheese</td>
<td>05</td>
</tr>
<tr>
<td>ND</td>
<td>-</td>
<td>milk</td>
<td>06</td>
</tr>
<tr>
<td>ND</td>
<td>+</td>
<td>milk</td>
<td>07</td>
</tr>
<tr>
<td>0.03ppb</td>
<td>+</td>
<td>milk</td>
<td>08</td>
</tr>
<tr>
<td>&lt;0.03ppb</td>
<td>+</td>
<td>cheese</td>
<td>09</td>
</tr>
<tr>
<td>0.02ppb</td>
<td>-</td>
<td>milk</td>
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<td>-</td>
<td>-</td>
<td>milk</td>
<td>11</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>cheese</td>
<td>12</td>
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</tbody>
</table>

Table 1: analysis of the presence of aflatoxin in the samples.

It is worth considering that isolated by *Penicillium* strains, *Aspergillus niger*, *Saccharomyces cerevisia*, *Saccharomyces paradoxus*, and *Yarrowia lipolytica*. From 85 samples of dairy products collected from Shiraz, molds were isolated from *Aspergillus niger* and *Penicillium* strains. some of the isolates had the ability of aflatoxin-bearing.
AFs-reducing traits of LABs

The results disclosed that 28 strains could completely eliminate fungi in the media. Notably, only 5 of the 28 strains tolerated the bile salts, coded as TD1/2, T21/2, T23/2, TD11, and LAX152. In the final step, for assessing 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: TD1/2, T23/2, and TD11. These three strains were selected as the final sample and referred for the molecular test of PCR with 16s rRNA.

The results of testing the six groups showed that the group without LABs, inoculated with AFM$_1$ and natamycin showed no reduction in the level of AFM$_1$ (0.5 ppm). Among the 5 groups with 5 strains of LABs, the mean level of AFM$_1$ 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 16s rRNA sequencing are shown in table 1.

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Number of nucleotides</th>
<th>Code of phylogenetic nomenclature</th>
<th>NCBI registration code</th>
<th>Search site of NCBI</th>
</tr>
</thead>
</table>

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

**Discussion**

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In this study, the *Penicillium* genus was the common dairy-contaminating genotype, followed by *Aspergillus niger*, *Saccharomyces cerevisia*, *Saccharomyces paradoxus*, and *Yarrowia lipolytica*. From 85 samples of dairy products collected from Shiraz, molds were isolated from *Aspergillus niger* and *Penicillium* strains. Most of isolates had the ability of aflatoxin-bearing.

Among 50 LABs, only 5 exhibited antifungal activity and bile salts resistance traits. Inoculation of 0.5 ppm AFM$_1$ to them was associated with significant amelioration in mean AFM$_1$ level. These three LABs were sequenced and uncovered as *L. fermentum*, *L. brevis*, and *E. faecium* strains existed.

Several surveys have exhibited 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 persons 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. We observed that 28 of 50 LABs exerted antifungal activity and 3 of them were highly potent for reducing AFM$_1$. In a previous study, 16/30 of LABs isolated from curd tolerated bile salts and acids (Praburajeshwar and Chandrakanth, 2017), highlighting our findings that not all LABs exert antifungal traits needing prior evaluation. 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). A study by Sadeghi et al., isolated *L. acidophilus* and *L. brevis* from traditional sourdough and examined inhibitory growth on *A. flavus* and reduction of AFB$_1$; a significant reduction in the AFB$_1$ level was observed and hence supposed these LABs (especially non–viable cells) as proper bioremediation for dairy products (Sadeghi et al., 2016). In another study, *L. brevis* was the most sensitive agent to reduce the AFM$_1$ level in milk (Verma et al., 2013). The studies (Verma et al., 2013; Sadeghi et al., 2016) highlight our results regarding the effect of *L. brevis* on AF levels. Fazeli isolated various LABs from sourdough and found significant reduction of AFB$_1$ by *L. casei*, *L. plantarum*, and *L. fermentum* (Fazeli et al., 2009). The results of this study highlighted the inhibitory effect of *L. fermentum* on AFs levels. Moreover, Nazhand et al, studied 20 LABs from which two strains of *E. faecium* had the highest
ability to eliminate Coumarin (similar to AFs) (Nazhand et al., 2017). These findings highlight that 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 LAB strains and AF types investigated varied among studies and the three strains supposed in the present study has been introduced here for the first time.

Various agents determine the efficiency of LAB on decrease of AFs and fungal development, such as the bacterial strain. Various bacteria employ different 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 preventing absorption of amino acids, leading to dissimilar antifungal potencies for various LABs (Perczak et al., 2018). Here, we studied the strains 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 the three properties. Furthermore, these three LABs had different potencies for reducing AFM$_1$ levels and *L. brevis* and *L. fermentum* had the highest reduction of AFM$_1$ levels. However, the best incubation period and temperature was supposed at about 48 h and 25–30°C (Dalié et al., 2010). A variety of incubation periods and temperatures have been used, for instance Fazeli et al., incubated LABs in the existence of AFB$_1$ at 37°C for 72 hrs observing that of AFB$_1$ removal by the strains differed being more at 72– vs. 24–hrs cultures (Fazeli et al., 2009). Noticeably, our samples were incubated at 22–25°C for 5 days and observed sufficient results. Furthermore, different inoculum amount of treatment has been reported as sufficient bacterial population for elimination of AFs in different studies (Abdelmotilib et al.; Hernandez-Mendoza et al., 2010). In a study by Sadeghi, the required bacterial population for *L. brevis* was 2×10$^3$ CFU (Sadeghi et al., 2016). Fazeli also reported 2×10$^3$ CFU as the sufficient amount for removal of AFs in *L. casei, L. plantarum, and L. fermentum* (Fazeli et al., 2009). In the present study, a mean value of 8×10$^3$ CFU: 3×10$^3$ CFU for *L. fermentum* and 5×10$^3$ cfu for *E. faecium* and the results showed 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**

We observed that 28/50 of LABs contained antifungal properties. For their applicability in human body conditions, we tested their resistance to bile salts and acids outlining that only three LABs had all the beneficial characteristics. The ability to reduce AFM$_1$ in comparison with
natamycin revealed that the LABs sequenced as *L. brevis*, *L. fermentum* and *E. faecium* strains had the highest ability to reduce AFM$_1$; eventually, the details of the three strains was registered in NCBI database. Accordingly, we proposed employment of these three strains to the traditional studied milk and cheese samples, which had high level of fungal contamination.

**Acknowledgments**

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**References**


