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ORIGINAL ARTICLE

Detoxification of aflatoxin M1 by probiotics *Saccharomyces boulardii*, *Lactobacillus casei*, and *Lactobacillus acidophilus* in reconstituted milk

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ABSTRACT

Aim: The current study aimed to remove aflatoxin from reconstituted milk by adding three probiotics, namely *Saccharomyces boulardii*, *Lactobacillus casei*, and *Lactobacillus acidophilus*.

Background: Aflatoxins are poisonous substances produced by certain kinds of fungi that are found naturally all over the world. They can contaminate food crops and pose a serious health threat to humans and livestock. Microbial detoxification is one method of eliminating aflatoxins, including aflatoxin M1.

Methods: For this purpose, about 109 and 107 cfu/ml of *S. boulardii, L.* casei, and *L. acidophilus* were inoculated into skim milk without aflatoxin M1. The samples were then spiked by aflatoxin M1 in concentrations of 0.5 and 0.75 ng/ml. The concentration of the aflatoxin residing in supernatant of milk samples after different storage times (30 and 90 minutes) and temperatures of 4 °C and 37 °C was measured by ELISA method, and the results were confirmed by HPLC.

Results: The results showed that the highest amount of aflatoxin M1 removal was related to *S. boulardii* (96.88 ± 3.79c) with a microbial density concentration of 109 cfu/ml and toxin concentration of 0.75 ng/ml at 37 °C for 90 minutes and then to *L. acidophilus* (71.46 ± 3.79b) with a microbial density concentration of 107 cfu/ml and toxin concentration 0.75 ng/ml at 4 °C for 90 minutes. Furthermore, the maximum level of AFM1 binding to 107 cfu/ml of *L. casei* with average binding percentages of 64.31 ± 3/79c was 0.75 ng/ml at 37 °C for 90 minutes.

Conclusion: The results revealed the possibility of using *S. boulardii* in combination with the selected probiotics of *L. casei* and *L. acidophilus* in the detoxification of AFM1-contaminated milk.

Keywords: Aflatoxin M1, Detoxification, *S. boulardii, L. casei, L. acidophilus.*

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Introduction

Aflatoxin is one of the most important basic fungal toxins produced through toxic fungi such as *Aspergillus parasiticus, Aspergillus flavus*, and *Aspergillus nomius* which grows on food, particularly cereals (1, 2). Livestock feed contaminated with such toxins creates a risk of liver cancer and other hazardous diseases (3). In livestock, aflatoxin B1 (AFB1) is metabolized in the liver and converted to aflatoxin M1 (AFM1), a compound believed to be less hazardous than AFB1, but which causes perilous diseases such as cancers and

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liver problems in milk consumers (4). This problem is mostly observed in livestock farms located in hot and humid areas (5). Not only food-storing countries, but also food-producing ones suffer from an aflatoxin crisis (6). Today, aflatoxins have become a global concern for food safety, human health, and their management, and the direct contact of humans (particularly the elderly and children) with AFM1 is one of the challenges and concerns in the fields of health and milk hygiene (7-9). Therefore, it is critical to maintain food hygiene and quality, particularly during storage (10). The International Agency for Research on Cancer (IARC) has recently classified AFM1 in group 1, the most dangerous carcinogenic group (11).

Until now, chemical, physical, or biological methods or compounds have been used to assess their ability to remove AF from milk and other dairy products (12-14). The success of probiotic-based detoxification has been confirmed by outcomes from recent studies, which were accomplished for milk and other dairy products as one of the dietary strategies to prevent humans from being contaminated with aflatoxin (13, 14). Some researchers have recognized the ability and role of lactobacilli and yeasts in binding to aflatoxin in milk and noted that *Saccharomycis boulardii* might be capable of removing AFM1 from milk mediums (2, 15). The aim of the present study was to determine the ability of commercially available strains of *Saccharomyces boulardii, Lactobacillus casei*, and *Lactobacillus acidophilus* at concentrations of 107 and 109 cfu/ml to bind AFM1 in skim milk contaminated with 0.5 and 0.75 ng/ml AFM1 at 30 and 90 min, and 4 ℃ and 37 ℃.

Methods

Microbial preparation

The probiotic medicinal yeast *Saccharomyces cerevisiae* HANSEN CBS 5926 (*S. boulardii* CNCM I-745) with a total of 2 x *109* cfu/ml of *bacteria* in a 250 mg Infloran capsule was purchased from Ardeypharm Germany, Gmbh and cultured in Sabaro Dextrose Agar (SDA) 2% medium at 25-30 °C for 48 h. It was subsequently relocated to the yeast nitrogen base (YNB) medium to be cultured at 25 °C in the logarithmic phase for 18 h. The lyophilized probiotics of *Lactobacillus casei* N;1608 and *Lactobacillus acidophilus* N;1643 were obtained from Iranian Research Organization from Science and Technology (ROST), Persian Type Culture Collection and cultured in De Man, Rogosa, and Sharpe agar (MRS) broth medium at 37 °C for 48 hr (16).

When the bacterial growth reached the logarithmic phase, a bacterial pellet was isolated after centrifugation at 3,500 X g for 10 min. A microbial suspension was prepared with PBS and was adjusted to a 3 McFarland standard at a concentration of 1 X 109 cfu/ml using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech Inc., USA) and the turbidity was measured at 600 nm (17). The 109 cfu/ml solution was diluted at 100:1 to obtain 107 dilutions for milk inoculation (18). Solutions were subjected to refrigerated centrifuge at 3500 g for 10 min, followed by collecting the pellet and discarding the supernatant. To prevent possible errors in the measurement of AFM1, bacterial cells were washed three times with 5 ml of sterile distilled water.

Milk preparation

According to the manufacturer's instructions, nonfat dry milk powder (Merck 115363, Germany) was characterized by *adding* distilled water (10: 1 V/W) to obtain a proper volume of solution for all 240 samples (each sample 100 µL) and evaluated by ELISA assay.

Aflatoxin solution and quantification

AFM1, produced from A. flavus, was purchased as an AFM1 analytical standard solution of 10 µg/mL in acetonitrile (SUPERCO 46319 Sigma-Aldrich) and diluted to 100 ng/ml using a ratio of acetonitrile: water of 25:75 (v/v). Two experimental concentrations of AFM1-contaminated skim milk were determined using a spectrophotometer (Ultrospec 2000): 0.50 ng/ml and 0.75 ng/ml. ELISA test was performed on 144 falcon tubes for the two concentrations. For the HPLC test, tubes were used for the 0.5 ng/ml and 0.75 ng/ml concentrations of AFM1-contaminated skim milk for 30 and 90 minutes (19).

Bacterial inoculation in to the milk

To produce a bacterial pellet, 1 ml of microbial suspension (107 and 109 cfu /ml of microbes) was centrifuged at 1000 rpm for 10 min. The pellet that formed at the tube bottom was washed twice with normal saline and then shaken gently to mix. One ml of sterile water was added to each pellet, and the pellet was added to 9 ml of pre-prepared contaminated milk.

The solution was stirred gently at 4 °C and 37 °C for 30 min and 90 min to mix well. After the specified times, the microtubes were centrifuged at 3500 × g for 10 min and the milk layer was analyzed to measure AFM1 levels (20).

Quantification of AFM1 by HPLC

Each sample was analyzed using the HPLC system (Breeze Separations Module, Waters, MA, USA) and attached to a two-pump solvent delivery system connected to a reverse phase column by an adjustable valve. The system was employed to measure the

Table 1. Average binding percentages of aflatoxin M1 to selected probiotics of *L. casei, L. acidophilus* and *S. boulardii* in the face of physicochemical conditions.

Microorganism	Microbial density	Temp	Toxin concentration (ng/ml)	Time (min)	Mean ± SE
L. casei	10^7 cfu /ml	4 °C	0.5	30	57.26±5.00a
				90	28.80±3.79b
			0.75	30	29.40±50.00b
				90	46.84±3.79c
		37 °C	0.5	30	24.60±5.00a
				90	32.40±3.79b
			0.75	30	63.86±5.00c
				90	64.31±3.79c
	10^9 cfu /ml	4 °C	0.5	30	31.13±5.00a
				90	42.06±3.79b
			0.75	30	59.91±5.00c
				90	56.57±3.79d
		37 °C	0.5	30	29.00±5.00a
				90	48.06±3.79b
			0.75	30	33.66±5.00c
				90	50.26±3.79b
L. acidophilus	10^7 cfu /ml	4 °C	0.5	30	33.06±5.00a
				90	42.26±3.79b
			0.75	30	49.11±5.00c
				90	56.15±3.79d
		37 °C	0.5	30	23.40±5.00a
				90	62.46±3.79b
			0.75	30	41.42±5.00c
				90	48.00±3.79d
	10^9 cfu /ml	4°C	0.5	30	36.73±5.00a
				90	71.46±3.79b
			0.75	30	42.00±5.00c
				90	54.97±3.79d
		37 °C	0.5	30	41.13±5.00a
				90	55.73±3.79b
			0.75	30	51.22±5.00b
				90	51.66±3.79b
S. boulardii	10^7 cfu /ml	4 °C	0.5	30	29.00±5.00a
				90	45.86±3.79b
			0.75	30	52.15±5.00c
				90	56.40±3.79d
		37 °C	0.5	30	43.93±5.00a
				90	28.06±3.79b
			0.75	30	43.06±5.00c
				90	45.93±3.79c
	10^9 cfu /ml	4 °C	0.5	30	35.73±5.00a
				90	11.00±3.79b
			0.75	30	44.71±5.00c
				90	58.86±3.79d
		37 °C	0.5	30	33.20±5.00a
				90	45.20±3.79b
			0.75	30	91.55±5.00c
				90	96.88±3.79d

amount of AFM1 remaining in the milk suspension contaminated with AFM1by an immunophylline column at 7,000 rpm at 4 °C for 10 min. This approach included a fully automatic fluorescence identifier (Breeze.417) with 365 and 465 nm wavelengths and an ODS Chromolith® column made of monolithic silica (2 × 4.6 × 100 mm) connected to a RP-18C terminal cover protective column (Merck, 102129). The system was used with the Empower Chromatography Data software. The percentage of AFM1 which was bound to the bacterial suspension was calculated using the following equation: AFM1 = (AFM1 of sample peak area / AFM1 of toxin control peak area) × 100 (21). All HPLC chemicals and reagents were purchased from Sigma-Oldrich, USA.

Results

The results of the binding values of AFM1 to *L. casei, L. acidophilus*, and *S. boulardii* in nonfat contaminated milk medium in the face of different selected physicochemical conditions are presented in Table 1.

The highest activity of *L. casei* in the removal of AFM1 toxin was observed at a concentration of 107 cfu/ml in 0.75 ng/ml and 37 °C (Figure 1). It was observed after 90 min of exposure (64.3% ± 31.79%) without a significant difference ($p > 0.05$) from the desired value in 30 min (63.86 ± 5).

Regardless of the AFM1 concentration and probiotic density, the highest marginal estimation percentage of AFM1 removal from the milk medium at 4 °C in initial minutes belonged to *L. acidophilus*, which gradually became the same for all three probiotics up to 90 minutes (Figure 2).

The lowest recorded dose of *L. casei* (24.5 ± 60) occurred at the same bacterial concentration and temperature with an AFM1 concentration of 0.5 and a minimum exposure time of 30 minutes (Table 1). However, this value was not significant compared to those removed by *L. acidophilus* and *S. boulardii*.

Unlike *L. casei, L. acidophilus* showed its highest AFM1 removal potential (71.3 ± 46.79) in milk medium at 4 °C (Fig. 2). Removal of AFM1 was increased by L. acidophilus (109 cfu /ml) from 30 to 90 min with decreases in the concentration and temperature. The lowest binding of AFM1 (23.5 ± 40.00) to this bacterium occurred in the first few minutes, which increased significantly (62.3 ± 46.79) with increases in temperature up to 90 min ($p < 0.05$).

Figure 2 shows that only the average marginal estimation of *L. acidophilus* increased in the elimination of AFM1 at 4 °C up to 90 minutes; the other two probiotics showed no increases at 4 °C.

S. boulardii had no such incremental pattern and followed no specific pattern. It had the greatest ability in AFM1 removal from milk medium (96.88 ± 3.79)

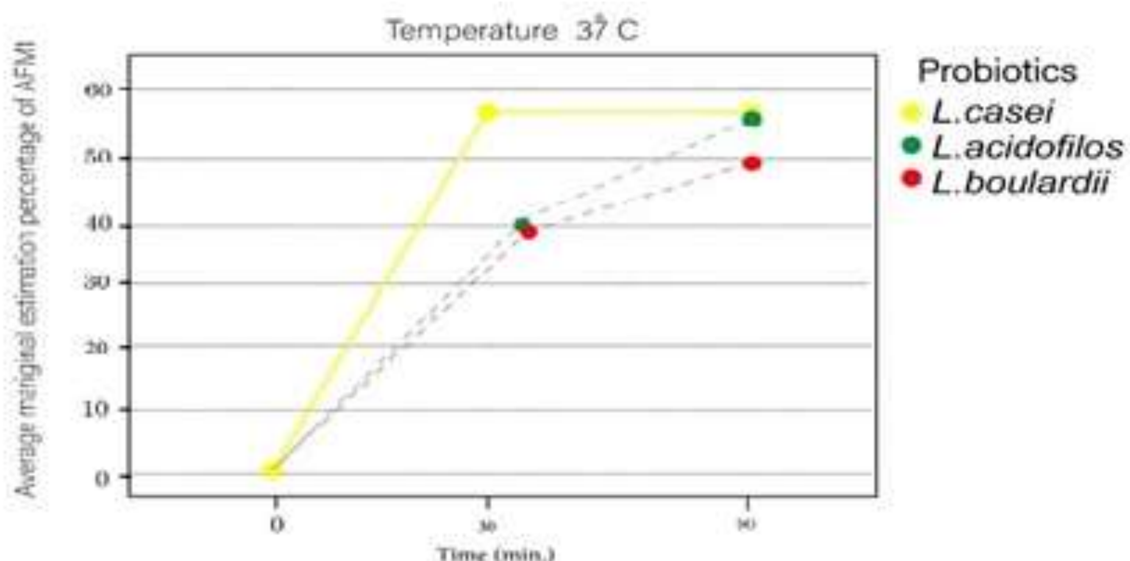

Figure 1. Average binding percentages of aflatoxin M1 (AFM1) to selected probiotics of *L. casei, L. acidophilus,* and *S. boulardii* at 37 °C.

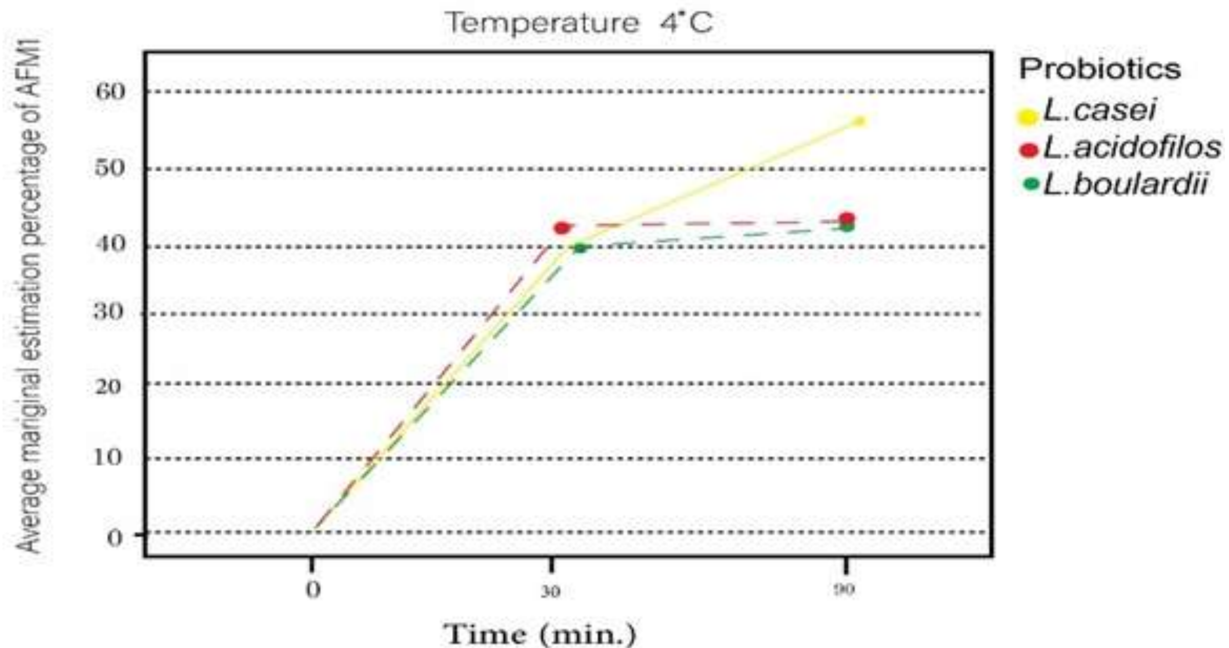

Figure 2. Average binding percentages of aflatoxin M1 to selected probiotics of *L. casei, L. acidophilus*, and *S. boulardii* at 4 °C

over time in the early hours (90 min) with increases in AFM1 concentration (0.75 ng/ml) and a concentration of 109 cfu /ml at 37 °C; however, binding declined with decreases in temperature.

The lowest value (29.5 ± 00.00) of AFM1 removal from milk medium by S. boulardii occurred at 4 °C with reductions in toxin concentration and yeast density.

Discussion

The current study investigated the removal of AFM1 from modified milk medium by adding three probiotics of *S. boulardii, L. casei*, and *L. acidophilus* in the face of some selected physicochemical parameters. Probiotic and lactic acid bacteria (LAB) have antimutagenic and anticarcinogenic effects and are able to bind dietary mutagens and carcinogens (22, 23). LAB strains from different origins can be used as starter cultures to reduce or remove AFM1 (15, 19, 22, 23). Traditional methods, such as cooking, freezing, or pressurizing, have little effect on aflatoxins (24). While chemical methods degrade toxins on the surface of contaminated food, the destruction inside entails a slow process (24).

Previous studies have demonstrated that AFM1 removal by probiotics has a potential application to reduce toxin concentrations to safe levels in milk (15, 22, 23).

The current results displayed that the highest amount of aflatoxin M1 removal was related to *S. boulardii* with a microbial density concentration of 109 cfu/ml and toxin concentration of 0.75 ng/ml at 37 °C for 90 minutes, and then to *L. acidophilus* with a microbial density concentration of 107 cfu/ml and toxin concentration of 0.75 ng/ml at 4 °C for 90 minutes.

The findings further showed that the tested probiotic strains of *S. boulardii, L. acidophilus*, and *L. casei* have immense potential in removing AFM1 and reducing its bioaccessibility in artificially contaminated skim milk as a model for a food matrix.

Similar to the current study, Panwar et al. worked on an in vitro digestion model and the AFM1 detoxification ability of probiotic Lactobacilli. They claimed that the selected probiotic strains could potentially be used to mitigate the toxic effects of AFM1 in contaminated milk and milk products and thereby enhance food safety (25).

In another study (26), *L. rhamnosus* GAF01 displayed its highest potency in AFM1 reduction at 107 cfu/ml, while in another research (27), the concentration of 107 cfu/ml for *Bifidobacterium animalis lactis* was the best offer for AFM1

detoxification at the 0.75 ng/ml level. In the current study, however, the two probiotics *S. boulardii* and *L. acidophilus* had the highest efficacy rates in binding to AFM1, respectively.

L. casei at high temperatures and low bacterial concentrations, however, had the greatest impact on toxins. The high binding of AFM1 at intense heat may be due to the denaturation of the cell wall proteins and increased hydrophobicity of the wall (28, 29).

The current research further revealed that AFM1 detoxification by *S. boulardii* was about 97% after 90 min, while in combination with *L. casei* and *L. acidophilus*, it AFM1 removal from milk medium reached 100%. Similar to the current study, Shahraki et al. (30) reported its AFM1 detoxification ability increased up to 99% in combination with LAB strains.

New studies have concluded that the majority of AFM1 binds to probiotics at early times of exposure (31-33). This result is consistent with the current study, as the highest removal of AFM1 occurred in the early hours of exposure and then decreased gradually.

The non-significant difference between AFM1 concentrations and exposure time in the ability of probiotics to remove the toxin is consistent with reports by Kabak et al. (15). However, AFM1 removal from milk medium depends on microbial strains and exposure time (32). This may be related to differences in AFM1 binding to cell walls of the different examined probiotics, which exhibited different results under the same conditions (32).

In the current study, an increase in AFM1 binding up to 90 min of exposure to the targeted probiotics and a reduction to 24 hours post-exposure indicated that the toxin binding sites on the cell walls of probiotics were gradually occupied after about 2 h of exposure, leaving no place for the binding of the remaining toxins (27). On the other hand, if the highest binding of AFM1 to microbial cell walls is considered to result from polysaccharides and peptidoglycans (2, 34), the higher percentage of AFM1 removal in the current research may be attributed to the high volume of these compounds in the outer layer of the *S. boulardii* cell wall.

Future studies will focus more on Iranian native probiotics and LAB strains and examine more variables, such as time, temperature, and bacterial concentrations, which were not possible in this study due to financial difficulties.

Conclusion

S. boulardii at a density of 107 cfu/ml at 37 °C could significantly reduce AFM1 in milk medium. AFM1 detoxification from milk medium was about 97% by *S. boulardii* after 90 min, while in combination with *L. casei* and *L. acidophilus*, it reached 100%. The results revealed the possibility of using some strains of LAB and *S. boulardii* in the detoxification of AFM1-contaminated milk. The application of this phenomenon in the removal of mycotoxins from contaminated food and feed is urgently needed to improve the safety of food and feed. Additional studies are needed to investigate the mechanisms involved in the removal process of toxins by LAB, aiming for its application in the dairy industry. The future trends are to identify the genetic characteristics that gave the probiotics and LAB strains the ability to remove AF.

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Conflict of interests

The authors declare that they have no conflict of interest.

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