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Detoxification of AFB1 by Yeasts Isolates from Kefir and Traditional Kefir-Like Products

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ABSTRACT

Background and objectives: Aflatoxin B1 (AFB1) is the most toxic aflatoxin produced by a large number of *Aspergillus* species. Successful detoxification of this toxin is an important attempt to improve community health. The aim of this study was to evaluate reducing effects of yeasts isolates from kefir and traditional kefir-like fermented beverages on AFB1 in a broth medium.

Methods: Polymerase chain reaction-sequencing was carried out to identify the yeast isolates from kefir and kefir-like beverages. Effects of the isolates on AFB1 adsorption and biotransformation in peptone dexterose broth medium were evaluated by using high performance liquid chromatography.

Results: *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* were isolated from kefir and kefir-like beverages and resulted in 46% and 53% AFB1 adsorption, respectively. The isolates 27Y and 2Y caused 7% toxin biotransformation, while 10% toxin biotransformation was achieved by the isolate 18Y.

Conclusion: Our results indicate that the yeast isolates from kefir and traditional kefir-like products can bind to and detoxify AFB1, thereby reducing its harmful effects.

Keywords: Kefir, Yeasts, Aflatoxin B1, Adsorption, Biotransformation.

INTRODUCTION

The growth of mold in various products and the production of aflatoxins not only causes human health problems, but also results in irreparable economic damage (1). Food contamination with aflatoxins is a common issue in tropical and subtropical regions of the world, especially in developing countries. Aflatoxin B1 (AFB1) is the most toxic aflatoxin and one of the most potent liver carcinogens known to date. This toxin is produced by a large number of *Aspergillus* species (2).

The use of antagonistic and harmless microorganisms to prevent or decrease the growth of *Aspergillus* species and subsequently to reduce aflatoxin production is one of the most important methods of biological food preservation (3). In this regard, lactic acid bacteria and yeasts play a key role (4). Detoxification and biological uptake of mycotoxins by *Saccharomyces cerevisiae* has already been shown. Isolated yeast cells belonging to different species including *S. cerevisiae* and *Candida cruzi* were tested for aflatoxin binding. Most yeast species absorb more than 15% of AFB1, and the toxin-binding ability is highly dependent on the species used (5, 6).

Kefir is one of the oldest fermented milk products, the origin of which is considered to be the Caucasus Mountains (7). This drink has been produced and consumed in the Caucasus for many years, and the lifespan of Caucasians who consume this substance is reported to be 110 to 150 years. Recent studies have revealed the beneficial effects of kefir on cancer and gastrointestinal disorders (8). Kefir is a lactic alcoholic beverage, and the fermenting agent of kefir milk, known as kefir grain, consists of casein and different microbial genera that are grouped together in gelatin-like colonies (8, 9). This fermented beverage is one of the main sources of lactic acid bacteria, such as *Lactobacillus kefiranofaciens*, *Lactococcus lactis*, *Lactobacillus helveticus*, *Lactobacillus kefiri*, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus plantarum*, and yeasts species including, *S. cerevisiae*, *Saccharomyces unisporus*, *Issatchenkia occidentalis*, and *Kluyveromyces marxianus* that cohabitate in a protein and polysaccharide (kefiran) matrix (10, 11).

Kefir not only modulates the immune system and improves the gastrointestinal function, but also exerts antioxidant, antimicrobial, and antitumor effects. Some studies have shown that lactic acid bacteria can significantly reduce the growth of *Aspergillus flavus* as well as AFB1 production (12). Some studies reported the anti-mycotoxin effects of kefir grains through binding to and reducing aflatoxins (12-14). Ansari et al. found that kefir grains are able to reduce 96.8% of aflatoxin G1 at a concentration of 20 µg/kg of pistachio nuts (13). In addition, kefir grains were reported to be able to bind up to 91.9% of aflatoxin M1 in milk at a rate of 0.5 µg/L (15).

There are few studies on the anti-mycotoxin effects of kefir and kefir-like traditional beverages in Iran. This study aimed to investigate effects of yeast isolates from kefir and kefir-like products on AFB1 in the Golestan Province, Northern Iran.

MATERIALS AND METHODS

Kefir and traditional kefir-like fermented beverages were obtained from sheep, camel, and mare milk in different areas of the Golestan Province, from May to February 2020. Grains were activated in commercial cow's milk with a specific content and incubated at 30 °C for 24 hours. The grains were then collected by sieving from the cultured milk and washed with mild sterile distilled water to remove the clotted milk. Then, the grains were re-inoculated into fresh milk and incubated under the same conditions. The milk was replaced every day for a week, and this step was repeated twice to obtain new adapted beans. All fermented products, including the traditional milk of sheep, camel, and mare were cultured using the same method.

Yeasts from kefir products were initially identified based on conventional microbiological tests and later confirmed by polymerase chain reaction (PCR)-sequencing. Data were recorded in the GenBank sequence database and then subjected to the Basic Local Alignment Search Tool (BLAST) In order to evaluate the ability of yeasts isolates from kefir and kefir-like beverages to biotransform or adsorb aflatoxin in a broth medium, first, stock standard solutions of (http://www.ncbi.nlm.nih.gov/BLAST/).

AFB1 (Sigma-Aldrich, USA) were prepared in methanol at 1 mg/mL and stored at −20 °C

until use. The broth medium was artificially contaminated with 100 ng/mL of AFB1 by adding appropriate amount of the stock standard. Falcon tubes containing 5 mL of AFB1-contaminated medium were prepared in triplicate to study the biotransformation and adsorption properties. The yeast isolates were propagated in 10 mL of yeast extract peptone dexterose (YPD) broth (QUELAB, Canada) at 30 °C for 3 days. Optical density (OD) was determined at 625 nm and adjusted to 0.08 with sterile broth medium. Next, YPD broth supplemented with 100 ng/mL AFB1 was prepared (13, 16). The yeasts were inoculated in YPD broth and added to tubes containing 5 mL of AFB1-contaminated medium as described before. The tubes were mixed and incubated aerobically at 30 °C for 24 hours. Three non-inoculated tubes containing only AFB1-contaminated broth were used as negative controls. After the incubation period, to measure AFB1 levels, tubes intended to test biotransformation were treated differently from those intended to test adsorption. To test biotransformation, 5 mL of YPD broth inoculated with the yeasts were directly added to test tubes (OD=0.08). After vigorous vortexing for 1 minute, the tubes were left to stand overnight at room temperature. The negative controls were also treated similarly. Then, the tubes were centrifuged at 9,000 g for 20 minutes, and the clear supernatant was transferred to new tubes and mixed with AFB1 (100 ng/mL). After vigorous vortexing for 1 minute, the tubes were left to stand overnight. Finally, all samples were filtered into clean 2 mL vials using a syringe filter (0.2 µm, Nylon) and preserved at −20 °C until high-performance liquid chromatography (HPLC) analysis (12, 16).

In order to evaluate effects of the yeast isolates on AFB1 adsorption, microbial cells were first isolated by centrifugation of 24-hour yeast cultures in YPD medium at 10,000 g for 10 minutes at 4 °C. Next, the cells were washed twice with 50 mM phosphate solution, suspended in sterile broth medium, and adjusted to about 10^8 CFU/mL (equivalent to 0.5 McFarland). One hundred ng/mL of AFB1 were then added to the microbial suspension. The suspension was incubated at 30 °C for 24 hours. The microbial cells were centrifuged at 10,000 g for 10 minutes, and the amount of aflatoxin in the supernatant was determined by using a HPLC instrument equipped with an ultraviolet detector (KNAUER. Germany) (12, 16). After washing the device and adjusting it to 365 nm, different dilutions of AFB1 and 30 µL of each sample were injected into the instrument's tank using a special syringe. The mobile phase consisting of a mixture of three solvents i.e. water/acetonitrile/methanol with volume ratios of 15/25/60 was passed through the column with velocity of 1 mL/min. Retention time for AFB1 in each sample was measured (~8.19 minutes), and peaks generated from the screen were viewed and saved in JPEG format. Finally, aflatoxin concentration in each sample was calculated according to the standard curve (16). In order to evaluate effects of the yeast isolates on the AFB1 biotransformation**,** 24-hour culture of yeast strains in YDP medium was centrifuged at 10,000 g for 10 minutes. The supernatant was passed through a 0.22 µm membrane filter. Then, 100 ng of AFB1 were mixed with 900 µL of this purified liquid to obtain a concentration of 100 ng/mL AFB1. The resulting mixture was incubated at 30 °C for 24 hours, and aflatoxin level was determined by the HPLC method as described above (12), (16). Results were expressed as mean values ± standard deviation (n=3). Statistical analysis of data was performed using the GraphPad Prism (version 7.00) for Windows. Two-way analysis of variance and Dunnett's post-hoc test were used to assess differences between effects of microorganisms and those of controls. All analyses were carried out at significance level of 0.05.

RESULTS

Five yeast isolates were recovered from the samples (Table 1). According to the PCR-sequencing results, the presence of *K. marxianus* and *S. cerevisiae* was confirmed. A selection of the fungal 26S rDNA sequences found in this study was submitted to NCBI and deposited in the GenBank data library under the following accession numbers: MZ466398, MZ466399, MZ466400, MZ466401, and MZ466402. After the overnight treatment with the yeast isolates, the amount of remaining AFB1 (100 ng/mL) ranged from 47±1.4 to 54±1.3 ng/mL in the broth tube. The treatment resulted in 46-53% toxin adsorption. In addition, toxin biotransformation of 7% was achieved using the isolates 27Y and 2Y, while 10% toxin biotransformation was achieved by the isolate 18Y.

Table 1- The identified yeast isolates from kefir products and grain samples

Yeast isolated on YPD medium	Origin	Closest relative	Identity (%) [a]	Accession number [b]
2Y	Milk (kefir)	*Kluyveromyces marxianus*	99.63	KJ491106.1
22Y	Grain	*Saccharomyces cerevisiae*	99.33	MG017576.1
25Y	Sheep kefir-like drink	*Kluyveromyces marxianus*	97.72	MH244202.1
18Y	Camel kefir-like drink	*Kluyveromyces marxianus*	99.45	MH244202.1
27Y	Mare kefir-like drink	*Kluyveromyces marxianus*	99.63	KY108055.1

[a] Percentage of nucleotide homology in the sequences obtained. The sequences of the nearest relatives were found by using the NCBI BLAST.
[b] Accession number of the sequence of the nearest relatives found by a BLAST in the GenBank database.

Table 2- Detoxification of AFB1 by yeast isolates from kefir and other fermented products.

Isolates	Origin	Remaining AFB1 residues in the medium (ng/mL)		AFB1 reduction [a]	
		Adsorption*	Biotransformation	Adsorption (%)	Biotransformation (%)
2Y	Milk (kefir)	50 ± 0.4	93 ± 1	~50	~7
22Y	Grain	54 ± 1.3	NP	~46	
25Y	Sheep kefir-like drink	51 ±1.4	NP	~49	NP
18Y	Camel kefir-like drink	47 ± 1.4	90 ± 1	~53	~10
27Y	Mare kefir-like drink	51 ± 09	93 ± 1	~49	~7

NP: Not performed.
[a] Percentage of AFB1 reduction by the yeast isolates from kefir and other fermented products.

DISCUSSION

Kefir and its grains have a diverse microbial community depending on the origin and environment of production (11). In this study, identification of yeasts isolates from kefir, kefir grains, and traditional fermented kefir-like beverages was performed by PCR-sequencing analysis. The results showed that the microbial diversity of the samples is similar to that of other studies (10, 11). However, the yeasts isolates in this study were different from other studies. This variation may be due to different origins of kefir grains and milks. To the best of our knowledge, the present study is the first to analyze three samples of traditional fermented kefir-like beverages from sheep, camel, and mare (9, 10, 17, 18).

Our results revealed the ability of yeasts in YDP broth adsorb AFB1 by about 50%, a figure that is consistent with the results obtained by Pizzolitto et al. (19) and Poloni et al. (20). Inconsistent with our findings, a study in Tunisia reported no binding potential for kefir-originated isolates in the YDP medium (12). Similar to our results, a study demonstrated that AFB1 concentrations were significantly reduced by 47–66% (average 58%) in kefir milk samples (14). However, Zolfaghari et al. found 30.46% and 5.40% AFB1 adsorption by *S. cerevisiae* isolates from yogurt and *K. marxianus* (*lactis*) isolates from cheese, respectively (6).

The bindings of toxins to different types of microorganisms may be attributed to the formation of a reversible complex between the microbial polysaccharides and the toxins (19-21). Lactic acid bacteria are able to produce exopolysaccharide kefiran, a water-soluble glucogalactane mainly produced by *Lactobacillus kefiranofaciens*. Kefiran forms a clear, flexible, homogeneous, and very thin layer with toxin-adsorbing properties. Similarly, the presence of β-(1,3 and 1,6)-D-glucan in the cell wall of *S. cerevisiae* and *K. marxianus* was found to be responsible for the observed adsorption effects of the two microbial strains (19, (19), (20).

CONCLUSION

Our study showed that yeasts isolates from kefir and traditional kefir-like products can bind to and detoxify AFB1, thereby reducing its harmful effects. The yeast isolates can be used as a microbial starter in the processing of fermented foods and production of novel dairy products as probiotics.

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Ethics approvals and consent to participate
This study was approved by the ethics committee of the Golestan University of Medical Sciences (ethical code: IR.GOUMS.REC.1400.404).

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest regarding publication of this article.

Authors' contributions
A.E performed the experiments. H. K supervised the research. F.N supervised the research and planned the experiments. A.A designed the experiments and helped in drafting the paper. H.R.J contributed to data analysis. All authors contributed equally to writing the final paper.

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