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The occurrence of aflatoxin M_1 in doogh, kefir, and kashk in Hamadan, Iran

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Abstract

Aflatoxin M_1 (AFM_1) is one of the mycotoxins found in milk and dairy products and is classified as a Group I carcinogen. In this study, samples of doogh (60), kefir (30), and kashk (20) were collected from Hamedan, Iran, and examined for AFM_1 contamination using the ELISA method. Ninety-two (83.64%) out of 110 samples were positive for AFM_1, with the highest amount of contamination in kashk (90%) and the lowest amount of contamination in kefir (73.33%). The level of AFM_1 in four (3.64%) of the samples (3 samples of doogh and 1 sample of kashk) was higher than in the European Union (0.05 µg/kg). However, all samples had AFM_1 lower than Iran's allowable limit, i.e., 0.1 µg/kg. Due to the low mean level of contamination in samples compared with the allowable limit, there is no concern for human health. However, it is necessary to monitor these products to decrease AFM_1 incidence.

Keywords: aflatoxin M_1; fermented dairy products; doogh; kefir; kashk.

Practical Application: The level of AFM_1 contamination in samples of doogh, kefir, and kashk was low, and there is no concern for human health.

1 Introduction

Aflatoxins are among the most critical fungal toxins that follow the growth of some *Aspergillus* species such as *A. flavus, A. parasitics,* and *A. nomius* produced in foodstuffs (Ismaiel et al., 2020; Bangar et al., 2022; Souza et al., 2021; Nourbakhsh & Tajbakhsh., 2021). Due to the toxicity and widespread distribution of aflatoxins in food and feed are among the most critical safety concerns (Nejad et al., 2019; Einolghozati et al., 2021; Heshmati et al., 2021b). Aflatoxins can lead to carcinogenesis of the liver, pancreas, kidneys, bladder, bones, and central nervous system, as well as, in the long run, cause anemia, malnutrition, delayed physical and mental development, and maturation of the nervous system (Mollayusefian et al., 2021; Mokhtarian et al., 2020; Pires et al., 2022).

There are different types of aflatoxins, such as aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2), although AFB_1 is the most toxic known mycotoxin (Sheng et al., 2021; Heshmati et al., 2021a). Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of AFB_1 created in animals that consumed foods containing AFB_1 and secreted in the milk (Nejad et al., 2019; Gonçalves et al., 2022; Heshmati et al., 2020; Jafari et al., 2021; Mohammadi et al., 2022). Therefore, AFM1 could be found in the animal's blood after 15 min of AFB1- pollute food ingestion and is then secreted in the milk of lactating animals (Assaf et al., 2019). The conversion rate of AFB_1 to AFM_1 was 0.3-6.2%. It depends on many factors, including animal health, swallowing speed, digestion rate, type of diet, liver biotransformation capacity, milk production, as well as environmental factors such as season, climate, Depends on the geographical location (Öztürk Yilmaz & Altinci, 2019; Nguyen et al., 2020; Mollayusefian et al., 2021). AFM_1 is classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 2002; Pellicer-Castell et al., 2020; Einolghozati et al., 2021). AFM_1 has a 2-10% carcinogenic potential of AFB_1 and binds to nucleic acid, causing DNA damage and ultimately leading to liver toxicity and carcinogenicity (Daou et al., 2020). Therefore, AFM_1 incidence in milk and dairy products has become one of the mighty concerns of food safety related to chemical risks (Pimpitak et al., 2020), especially for vulnerable groups such as children and older adults (Gonçalves et al., 2021). According to its Harms and stability against pasteurization, heat inactivation, and food processing measures, it is vital to apply routine monitoring measures of AFM_1 in dairy products (Ahmadi, 2020).

To protect public health, rules for permissible levels of AFM_1 in milk and dairy products have been established in several regions, varying from one country to another countries (Vaz et al., 2020; Souza et al., 2021; Lima et al., 2022; Turna & Wu, 2021). For example, the AFM_1 allowable limit in milk, doogh, and kefir, according to the Iranian National Standardization Organization (ISNO), Iran was 0.1 µg/kg. At the same time, the EU commission considered it 0.05 µg/kg (European Union, 2010; Iranian National Standardization Organization, 2020).

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Milk and its products help balance the human diet at different ages. As AFM_1 is resistant to thermal processes such as pasteurization, ultra-high temperature processing, and other processing methods, it may also be found in dairy products. Traditional fermented products such as kefir and doogh are also exposed to AFM_1 contamination (Vasconcelos et al., 2020; Souza et al., 2021).

kashk is one of the conventional fermented dairy foodstuffs with a high nutritional value and is rich in protein, calcium, and phosphorus (Mason et al., 2016). Today, kashk is also produced industrially and produced and consumed in liquid and dry forms (Pourjoula et al., 2020).

Doogh is a traditional dairy drink made from yogurt and is very popular in Iran and other Middle Eastern countries (Sarlak et al., 2017b). Yogurt, drinking water and sodium chloride, and yogurt starter culture are the main components of doogh (Fallah et al., 2011). Doogh produced in Iran is exported to neighboring countries like Turkey, Azerbaijan, Afghanistan, the Middle East, and Central Asia.

Kefir is a fermented milk product that is a rich source of amino acids, vitamins (B_2, B_{12}, K, A, D), minerals (calcium, phosphorus, magnesium), and enzymes (Bashiti et al., 2019). Kefir has many therapeutic effects: strengthening the immune system, inhibiting tumor growth, preventing aging and allergies, lowering cholesterol, and treating sleep disorders (Gaware et al., 2011; Sarlak et al., 2017a). Traditionally, kefir is obtained by the fermentation of kefir grains of varying sizes (1-4 cm long) and varying from white-to-light yellow with irregular, cauliflower appearance, gelatinous and sticky structures (Mitra & Ghosh, 2020; Biçer et al., 2021). Cow, goat, camel, sheep, or buffalo milk can be used to produce kefir (Larosa et al., 2021). Kefir grains differ from fermented products and are sensitive to multiple variations, which may derive from invoices such as the origin and storage of kefir grains, milk type (substrate), the microbiological composition of grains, processing conditions, grain/milk ratio, and environmental conditions like fermentation time and temperature (Almeida Brasiel et al., 2021).

There are various reports about the incidence of AFM_1 in milk and some dairy products in Iran and other countries (Bahrami et al., 2016; Nejad et al., 2019; Daou et al., 2020; Nejad et al., 2019; Souza et al., 2021; Khaneghah et al., 2021; Mollayusefian et al., 2021; Turna & Wu, 2021). However, low information was found regarding this mycotoxin in dairy products such as kashk, doogh, and kefir, especially in western Iran. This study aimed to investigate the level of AFM_1 in kashk, doogh, and kefir and assess its change during storage.

2 Materials and methods

2.1 Materials

Dichloromethane and methanol, and other used chemicals were bought by Merck (Darmstadt, Germany). ELISA kit was supplied by R-Biopharm (Darmstadt, Germany).

2.2 Sampling

In this study, 110 samples of traditional dairy products (doogh, kefir, and kashk) were collected from Hamadan in western Iran in June 2021 and transferred to the laboratory with a cold chain and evaluated for AFM_1. LEISA determined the concentration of AFM_1 during two stages, i.e., after collection and one month after sample collection and storage in the refrigerator, to indicate AFM_1 change during storage.

2.3 Preparation of samples for AFM_1 determination

All procedures utilized in the current study were accepted by the Ethics Committee of Hamadan University of Medical Sciences, Iran. IR.UMSHA.REC.1396.617.

For doogh and kefir samples, 10 mL of each sample was centrifuged at 1372 g for 10 min. By removing supernatant from t creamy layer, 100 µL of the bottom layer was removed using a pipette and added to the wells.

For kashk samples, the samples were mixed and completely homogeneous, then 2 g of the sample was poured into a Falcon tube (15 mL), and 8 mL of dichloromethane was added and centrifuged for 10 min at 10 °C at 1792 g. After removing the top creamy layer, 4 mL of the bottom layer was removed using a pipette, transferred to a falcon tube, and placed at 60 °C to evaporate the solvent. Then it was added to the falcon tube containing distilled water (one mL), methanol (one mL), and hexane (3 mL) and stirred thoroughly, and centrifuged (15 min at 15 °C, 1372 g.) Then the methanol-water layer (bottom layer) was removed and used for AFM_1 measurement (Bahrami et al., 2016).

2.4 AFM_1 measurement

The sample AFM_1 analysis was performed according to ELISA kit instructions. All reagents were placed at ambient temperature. First, 100 µL of antibody in duplicate was poured into wells and mixed gently by manually shaking the plate and incubating for 15 minutes at room temperature. The liquid into the wells was poured out and washed wells with washing buffer. The liquid was poured out. The washing procedure was repeated two times. Then, the samples or standards (100 µL) were transferred to the wells, mixed by shaking manually for 30 s, and incubated (30 min) at room temperature in the dark. The liquid was poured out, and the well was washed three times. Then, 100 µl of conjugate solution were added, mixed, incubated, and washed. In the final, 100 µl of substrate/chromogen (100 µL) was poured into a well, mixed, and incubated (15 min) at room temperature in the dark. The stop solution was added mixed. After 15 min, the absorbance of the sample was measured with an ELISA reader (BioRad, CA, USA) at a wavelength of 450 nm.

2.5 Risk assessment

For risk assessment of intake AFM_1 and the probability of liver cancer created by this mycotoxin, the estimated daily intake [(EDI) and hazard index (HI) were calculated by the following Equations 1 and 2:

$$EDI\ \left(ng/kg\ bw/day\right) = AFM_1 average\ \left(\mu g/kg\right) \times daily\ consumption\ of\ dairy\ products\ \left(kg/day\right)/\backslash\ bw\ \left(kg\right) \quad (1)$$

$$HI = EDI / \text{ the tolerance daily intake } (TDI) \quad (2)$$

According to the previous study, the daily consumption of dairy products in Iran was approximately 0.192 kg/day (Nejad et al., 2019). The average bw for an adult Iranian person was considered 70 kg. Also, TDI was considered 0.2 ng/kg/day as suggested by Kuiper-Goodman (Kuiper-Goodman, 1990).

2.6 Statistical analysis

Statistical Analysis was performed by SPSS Statistics (IBM SPSS Statistics for Windows, Version 20.0, NY, USA). The mean and standard deviation concentrations of AFM_1 in milk samples were calculated and reported. AFM_1 reduction amount during storage was determined. A one-sample t-test was applied to determine the significant difference between the average concentration of samples AFM_1 with the maximum permissible limit according to ISIRI (0.1 µg/kg) and European Union (0.05 µg/kg) regulation. An Independent T-test was applied to determine the significant difference between AFM_1 in the initial sample and the stored one. Differences between values were considered significant at $P \leq 0.05$.

3 Result and discussion

shown in Table 1, 82 (78.12%) out of 110 samples were contaminated with AFM_1 in different concentrations between 0.005-0.085 µg/kg with mean contamination of 0.018 ± 0.016 µg/kg, and 4 samples (3.64%) contained AFM_1 higher than the limit (0.05 µg/kg) premised in the European Union (2010). However, all samples had AFM_1 lower than the value accepted in Iran, i.e., 0.1 µg/kg AFM_1 (Iranian National Standardization Organization, 2020). AFM_1 analysis in each fermentation product is described separately below.

3.1 The incidence of AFM_1 in doogh

As shown in Table 1, the incidence of AFM_1 contamination in doogh samples was 86.67%, ranging from 0.005 to 0.085 µg/kg. Three (5%) samples contained AFM_1 contamination at a higher level than the allowable limit, according to the European Union (0.05 µg/kg). However, according to Iran regulation, all samples had AFM_1 lower than the acceptable value, i.e., 0.1 µg/kg. Comparing the kashk samples, the mean AFM_1 in doogh specimens was lower. AFM_1 binds to milk proteins, especially casein (Vaz et al., 2020), and since 50% of doogh is water, therefore casein amount of doogh was less than kashk. As a result, the amount of AFM_1 in the doogh decreases (Bahrami et al., 2016). In a study conducted by Bahrami et al. (2016) in Iran, the incidence of AFM_1 in 44 doogh samples was 13.6% lower than our findings (86.67%). However, the mean of this mycotoxin in the current study (0.018 ± 0.017 µg/kg) was higher than mentioned (9 ± 0.9 ng/kg or 0.009 ± 0.0009 µg/kg) report (Bahrami et al., 2016).

In another study conducted by Tabari et al. (2013) in Iran, the level of aflatoxin M_1 in traditional (n=115) and industrial pasteurized doogh (n=110) was investigated. AFM_1 was found in 83 (72.1%) traditional doogh samples (mean: 52.3 ng/L or 0.0523 µg/L) and 68 (61.8%) industrial doogh samples (mean: 46.4 ng/L or 0.0464 µg/L). In addition, fifteen (12.8%) out of traditional samples and 12 (10.8%) of industrial ones had greater AFM_1 than the allowable limit (0.05 µg/kg) in the EU (European Union, 2010). Also, 5 (2.2%) of total samples had AFM_1 more than the permissible limit (0.1 µg/L) in Iran standard (Iranian National Standardization Organization, 2020). Therefore, the incidence of AFM_1 in this study was lower than ours, although the mean and unacceptable sample in the mentioned study was greater (Tabari et al., 2013).

In the study performed by Fallah et al. (2011) in Iran, the incidence and mean of AFM_1 contamination in industrial (n=71, manufactured from cow milk) and traditional (n=6, prepared from goat and sheep milk) doogh samples was 22.5% and 0.007% µg/L and 13.8% and 0.003 µg/L, respectively. The incidence and mean of AFM_1 in the mentioned study were lower than this. However, the incident of samples containing AFM_1 greater than 50 µg/kg was almost similar (5% in our study and 4.2% by Fallah et al., 2011).

3.2 The incidence of AFM_1 in kefir

AFM_1 was observed in 22 samples (73.33%) out of 30 kefir samples (range 0.006-0.050 µg/kg, mean 0.014 ± 0.015 µg/kg). AFM_1 contamination level in all kefir samples was to European Union (0.05 µg/kg) and Iran standards (0.1 µg/kg). In general, the AFM_1 contamination concentration in kefir samples was lower than in doogh and kashk samples, and AFM_1 concentration in 26.67% of samples was <0.005 µg/kg. According to previous studies, probiotic stains into kefir could bind to aflatoxins and decrease them (Taheur et al., 2020, 2021; Emadi et al., 2021).

3.3 The incidence of AFM_1 in kashk

Eighteen (90%) of 20 analyzed kashk specimens contained AFM_1 with a mean of 0.021 ± 0.015 µg/kg. The concentration of AFM_1 in 85% of the samples was 0.005-0.05 µg/kg. 85% of the samples had AFM_1 lower than the accepted value following European Union (0.05 µg/kg), although one kashk sample had AFM_1 contamination more than the allowable limit of 0.05 µg/kg.

Table 1. The incidence and level (µg/kg) of AFM_1 in fermented dairy samples of Hamadan province, Iran.

Sample type	Number of samples	Positive	Mean	Standard deviation	<0.005	0.005-0.050	0.051-0.1	>0.1	Range in positive samples
Doogh	60	52 (86.67%)	0.018	0.017	9 (15%)	48 (80%)	3 (5%)	0	0.005-0.085
Kefir	30	22 (73.33%)	0.014	0.015	8 (26.67%)	22 (73.33%)	0	0	0.006-0.050
Kashk	20	18 (90%)	0.021	0.015	2 (10%)	17 (85%)	1 (5%)	0	0.005-0.056
Total	110	82 (78.12%)	0.018	0.016	19 (17.27%)	87 (79.09%)	4 (3.64%)	0	0.005-0.085

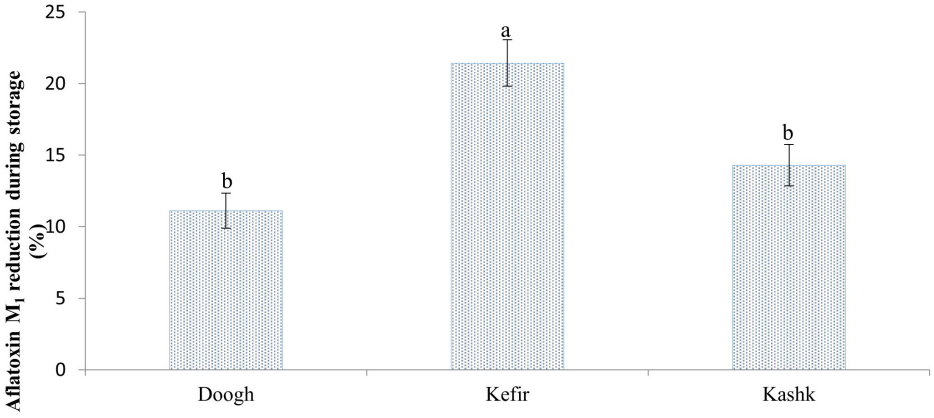

Figure 1. Aflatoxin M_1 (AFM_1) reduction amount during storage.

The level of AFM_1 in all samples was less than Iranian standards (0.01 µg/kg). Among the samples analyzed in this study, the most AFM_1 contamination was observed in this product. High kashk contamination can be because the kashk of our product is concentrated (Fallah et al., 2011).

There have been several reports of AFM_1 contamination in kashk. Bahrami et al. (2016) reported 14 (35%) out of 40 kashk samples (Bahrami et al., 2016). The mean concentrations of AFM_1 with mean 62.1 ng/L or 0.0621 µg/L). Fallah et al. (2011) studied AFM_1 in 64 industrial kashk and 61 traditional kashk in Iran. The prevalence of AFM_1 in industrial and traditional kashk was 53.1% (mean: 0.080 µg/kg) and 31.2% (mean: 0.053 µg/kg), respectively (Fallah et al., 2011), which was less than our study. Mason et al. (2016) reported the average concentration of AFM_1 in traditional Iranian kashk as 0.118 µg/kg (Mason et al., 2016), which was higher than our study. Amirpour et al. (2015) found that 29 (90.62%) out of 32 industrial liquid Kashk samples (mean: were contaminated with AFM_1 (60.17 ± 75.48 ppt or 0.06017 µg/kg). The incidence of AFM_1 in this study is almost similar to ours, although the mean AFM_1 in the mentioned study was greater (Amirpour et al., 2015). The difference in the prevalence of AFM_1 contamination in different studies is due to animal physiology, geographical location, type of forage, and AFM_1 measurement different methods.

3.4 AFM_1 change during storage

After two months of storage, the mean AFM_1 content of doogh, kefir, and kashk was 0.016, 0.011, and 0.018 µg/kg, respectively. Compared with the initial sample, mean AFM_1 in samples stored for two months was decreased by 11.11, 21.43, and 14.29%, respectively (Figure 1).

The AFM_1 reduction in the fermented products during cold storage could be related to the decrease in pH, the occurrence of lactic acid bacteria and the production of lactic acid and organic acids by these bacteria, and other by-products of fermentation, including aldehydes, amino acids, volatile fatty acids and peptides (Govaris et al., 2002). AFM_1 concentration, storage temperature and time, differences in the type of starter culture used to produce dairy products, variation in milk composition, and milk contamination method may result in the variation in AFM_1 decrease value in different fermented dairy products (Adibpour et al., 2016).

3.5 Risk assessment of AFM_1 intake through dairy products

EDI of AFM_1 intake through analyzed dairy products was 0.0409 ng/kg bw/day. The value of EDI in this study was lower the previous studies (Bahrami et al., 2016; Nejad et al., 2019). As the obtained HI in this study (0.247) was lower than 1, demonstrating this fact that the consumption of analyzed dairy products such as doogh, kefir, and kashk had no potential risk for creating liver cancer among Iranian consumers. The HI value reported by Nejad et al. (2019) in Iran (0.535) and Milićević et al. (2017) in Serbia (11.78 for males and 11.52 for females) were greater than the current study (Milićević et al., 2017; Nejad et al., 2019).

4 Conclusion

Our finding indicated a high incidence of AFM_1 in doogh, kefir, and kashk samples. The mean of AFM_1 in all analyzed samples was lower than EU and Iran standards; therefore, the AFM_1 value in these products had no concern for human health. However, it is necessary to monitor these products to decrease AFM_1 incidence. The inhibition of controlling animal feed and inhabitation of molding of these products could be a suitable method for decreasing AFM_1 in milk.

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