Research Paper

Aflatoxin M1 Contamination in Milk From North Khorasan Province: Raw vs Pasteurized vs Sterilized

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ABSTRACT

Background: The presence of aflatoxin M1, a toxic, mutagenic, and carcinogenic substance, poses significant health risks. Hence, this study assesses the levels of aflatoxin M1 in raw, pasteurized, and sterilized milk obtained from the North Khorasan Province, Iran, and evaluates its potential impact on consumer health.

Methods: This descriptive-analytical study randomly collected 189 raw milk samples. Additionally, 70 pasteurized and sterilized milk samples from supermarkets in the North Khorasan Province, Iran, were included. All samples were assessed for aflatoxin M1 contamination using the enzyme-linked immunosorbent assay method. Meanwhile, statistical analysis (analysis of variance) was performed on the results.

Results: The Mean±SD concentration of aflatoxin M1 in raw milk from the North Khorasan Province, Iran, was 18.44±34.6 ng/L, while pasteurized and sterilized milk had a higher mean concentration of 42.8±21.54 ng/L. The lowest concentration was found in Bojnord City, Iran, at 9.30±8.91 ng/L, while the highest concentration was detected in Jajarm at 30.70±80.50 ng/L. The mean contamination of aflatoxin M1 was not statistically significant (P=0.42) in most cities of the province. Meanwhile, 6.34% of raw milk and 11.4% of pasteurized milk samples had higher levels of aflatoxin M1 than the maximum limit recommended by the Iran National Standard Organization (INSO) (100 ng/L). However, none of the milk samples exceeded the permissible limit set by the Veterinary Organization of the country (500 ng/L).

Conclusion: The mean concentration of aflatoxin M1 in raw and pasteurized milk from the North Khorasan Province is lower than the European Union (EU) standard of 50 ng/L and the approved limit set by the country's Veterinary Organization (500 ng/L). Therefore, it is not considered a significant threat to the health of adults.

Keywords: Aflatoxin M1, Milk, Food contamination, ELISA

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Introduction

Mycotoxins are natural contaminants that can be found in food and agricultural products. They are created by molds and fungi, with certain types of fungi from the *Aspergillus* genus as the main culprits. Specifically, *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* are known to produce these toxins. According to the Food and Agriculture Organization (FDA), around 25% of agricultural products worldwide are affected by mycotoxin contamination [1]. Fungi tend to thrive in hot and humid environments and can produce mycotoxins. Among the mycotoxins identified in nature, at least 18 types of aflatoxins are known, with aflatoxin B1, B2, G1, and G2 being particularly significant. When animals consume feed contaminated with aflatoxin B1, the toxin is metabolized in the liver and converted into aflatoxin M1 (AFM1). This mycotoxin is then excreted in milk and can harm human organs, particularly the digestive system and liver. Depending on its concentration and duration, AFM1 can cause mutagenesis, carcinogenesis, and immune system suppression [2]. Meanwhile, AFM1 resists common heat treatment methods like pasteurization, sterilization, and autoclaving [3]. AFM1's high resistance to freezing and fermentation makes it a concern in producing raw animal products like yogurt, buttermilk, and cheese, which can be made using contaminated milk [4]. Although children are more susceptible to the health risks associated with AFM1-contaminated milk, dairy products are also important sources of nutrients essential for human growth and well-being, especially in children [5]. The International Agency for Research on Cancer recently classified aflatoxin M1 as a Group 1 carcinogen, highlighting the significant health risks this mycotoxin poses [6]. To safeguard the safety and health of human societies, government institutions must conduct rigorous monitoring of food and livestock products. To mitigate the adverse effects of AFM1, many countries and international organizations have established maximum permissible limits for this mycotoxin in milk and dairy products. Various chromatography techniques, including thin-layer chromatography, liquid chromatography, high-performance liquid chromatography, and enzyme-linked immunosorbent assay (ELISA), are available for detecting and analyzing AFM1 in food, milk, and dairy products. ELISA is a particularly advantageous method, offering reduced test times, simple sample preparation and extraction, low cost, and high sensitivity [7]. Several studies have investigated the levels of AFM1 in raw and pasteurized milk in Iran, including in North Khorasan Province, Iran; however, a comprehensive study of this issue has yet to be conducted in the region [7-10]. Given that traditional livestock management practices are common in the province and may not prioritize health standards, it is necessary to implement specific management measures and prevention programs. Therefore, this study assesses the levels of AFM1 in raw, pasteurized, and sterilized milk produced in the cities within North Khorasan Province, Iran.

Methods

This study is a descriptive-analytical investigation conducted in North Khorasan Province, Iran. The minimum sample size was calculated using the method described by Karimi et al. in 2007, considering a contamination rate of 60% (P=0.6), a confidence level of 95%, and an acceptable error value of 5%, total sample size calculated was 258 [11] (Equation 1):

1. $$n = \frac{z^2(1-\frac{\alpha}{2}) \times p(1-p)}{d^2}$$

The samples were divided into two groups as follows: Raw and pasteurized milk. The sampling method in this study is multi-stage. First, we consider each city as a stratum, and sampling is done from all strata. In the next stage of sampling, the cluster sampling method was used, each industrial livestock unit was considered a cluster, and each unit of milk collection center and milk tanker was taken as a cluster. In addition, each traditional livestock was considered a cluster. Based on the number of traditional livestock in each village, the number of samples was determined. A total of 189 raw milk samples were collected randomly in 6 months from various sources in the cities of Bojnurd, Esfarayen, Shirvan, Jajarm, Mane and Samalqan, Garmeh, Faruj, and Raz and Jargalan. This study was conducted in the summer and autumn of 2022. These seasons are suitable for the growth of molds and fungi. The samples were obtained from 39 raw milk collection centers, 57 milk trucks, 62 traditional livestock farms, and 31 industrial livestock farms. Additionally, 69 samples of pasteurized and sterilized milk produced by nine milk factory brands were collected from supermarkets in North Khorasan Province. The sampling was conducted during the summer and autumn seasons. Before collecting the samples, all necessary materials and equipment were washed with detergent and sterilized by autoclaving. The collected milk samples were transported to the laboratory under sterile conditions and in an ice-cooled environment.

To prepare the samples, all chilled milk samples were initially centrifuged at 3500 rounds per min to obtain skimmed milk while completely removing the upper creamy layer. Subsequently, 100 µL of skimmed milk samples were extracted for AFM1 analysis.

In this experiment, an ELISA kit (Veratox, Neogen) with a sensitivity of 5 parts per trillion was employed to measure the concentration of AFM1 in milk samples. The test was performed according to the instructions provided by the manufacturer of the kit. Specifically, 50 µL of each standard (0, 250, 500, 1000, and 2000 ng/L) was added to 50 µL of skimmed milk samples in each well. Subsequently, 50 µL of conjugation and 50 µL of antibody were added to each well in sequence. To ensure thorough mixing of all contents in each well, the kit was manually moved several times in different directions. After allowing the kit to stand at room temperature for 10 min, the test results were recorded. After 10 min, the contents of the kit were removed, and each well was washed three times with distilled water. The kit was then inverted to drain the distilled water and ensure complete drying of the wells. Next, 100 µL of chromogen solution was added to each well, after which the kit was manually agitated in different directions to thoroughly mix the contents of each well. The kit was then placed in a dark environment for 5 min. After 5 min, 100 µL of stopping solution was added to each well, and the kit was agitated before measuring the light absorption using an ELISA reader at a wavelength of 650 nm. The concentrations of AFM1 were calculated by drawing a curve based on the recorded results. Additionally, the ELISA method was utilized in this study to identify positive samples for the presence of AFM1 in mastitis milk samples.

AFM1 rapid test kits are commonly used for the rapid detection of aflatoxin M1 in raw milk and milk products. In this study, all raw milk samples were assessed using AFM1 rapid test kits (Milkguard Rapid Test Kit for Aflatoxin M1, Kwinbon Biotech, China). According to the manufacturer's instructions, the sensitivity of this kit was 0.1 parts per billion. The obtained results were analyzed using the SPSS software, version 22 and the Tukey test (analysis of variance) with a significance level of $P<0.05$.

Results

Over 6 months during the summer and autumn of 2023, a total of 189 raw milk samples and 69 samples of pasteurized and sterilized milk were collected from supermarkets in the province. The results showed that AFM1 was detected in 93.65% of raw milk samples and in 42% of pasteurized and sterilized milk samples, with concentrations ranging between 1-50 ng/L. The Mean±SD of AFM1 concentrations in raw and pasteurized milk samples varied across the cities of Bojnurd, Esfarayen, Shirvan, Jajarm, Mane and Samalqan, Garmeh, Faruj, and Raz and Jarga-

Table 1. AFM1 samples in raw and pasteurized milk in the cities of North Khorasan Province, Iran

Location	No.	Mean	Standard Deviation	Standard Error	95% Confidence Interval for Mean		Minimum	Maximum	P
					Lower Bound	Upper Bound			
Esfarayen	26	17.31	21.73	4.26	8.54	26.09	0.00	113.78	
Bojnurd	27	8.92	9.30	1.79	5.24	12.60	0.00	30.03	
Dairy companies	69	21.55	42.80	5.15	11.27	31.83	0.00	254.18	
Jajarm	26	30.71	80.50	15.79	-1.81	63.22	0.00	416.97	0.42
Raz	20	13.74	16.13	3.61	6.19	21.29	0.00	45.29	
Shirvan	22	21.17	17.70	3.77	13.32	29.02	2.41	73.85	
Faroj	28	26.73	24.34	4.60	17.30	36.17	0.00	79.40	
Garmeh	20	13.35	18.09	4.05	4.89	21.82	0.00	83.68	
Mane and Semelghan	20	11.27	14.44	3.23	4.52	18.03	0.00	50.19	
Total	258	19.22	36.87	2.30	14.70	23.74	0.00	416.97	

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lan, as well as different dairy factories, with the values of 17.31±21.72, 8.91±9.30, 30.70±80.50, 13.73±16.12, 17.69±21.16, 24.33±26.73, 18.09±13.35, 14.43±11.27, and 42.80±21.54 ng/L, respectively (Table 1).

Bojnord had a higher mean level of AFM1 (8.91±9.30), while Jajarm had a lower mean AFM1 level (80.50±30.70). However, statistical analysis did not reveal any significant differences in AFM1 levels between the different cities or pasteurized milk (P=0.42).

The mean AFM1 concentration was lower in milk collection centers (11.40±18.6) and higher in industrial farms (26.92±21.47); however, this difference was not statistically significant (P=0.94).

The mean concentration of AFM1 in milk samples from North Khorasan Province (36.86±19.21) was lower than the European standard. However, in terms of relative frequency, the level of contamination in some cities, such as Esfrain and Jajarm, exceeded the national standard of Iran by 3.8%. However, all the milk samples analyzed in this study were below the permissible limit set by the Iranian Veterinary Organization (500 ng/L), as shown in Tables 2 and 3. On the other hand, the average concentration of AFM1 in pasteurized and sterilized milk samples from the province was higher than both the European and Iranian standards, with a mean concentration of 42.8±21.54 ng/L. The relative frequency of samples exceeding the European and Iranian standards was 11.4% and 5.7%, respectively (Table 4). In this study, the sensitivity of the AFM1 rapid test kit for the detection of AFM1 in raw milk was evaluated and compared to the ELISA method. The results indicated that this kit lacked the necessary sensitivity to detect AFM1 in the concentration range of 50-500 ng/L; therefore, it did not exhibit sufficient efficiency for use in this study.

Discussion

The potential health risks associated with AFM1 in milk and dairy products are significant. In this study, 93.65% of raw milk and 87.14% of pasteurized and sterilized milk were contaminated with AFM1 in the range of 0-50 ng/L, which according to studies conducted in the European Union (EU), are within the allowable limit of aflatoxin levels and do not have adverse effects on the health of adults [12]. The remaining milk that has higher contamination levels needs further analysis. To accurately assess and interpret these findings, it is essential to establish a recognized limit for AFM1 in the country. The existence of multiple standards across the country has complicated this analysis. Many researchers in Iran have compared and contrasted the EU's standard limit of 50 ng/L [12] with the National Standard Organization's limit of 100 ng/L [13]. These comparisons have raised concerns about the high levels of AFM1 in milk and underscore the need for a consistent and effective

Table 2. AFM1 samples in milk by raw milk location of collection

Location	No.	Mean±SD	95% Confidence Interval for Mean		Minimum	Maximum	%		P
			Lower Bound	Upper Bound			Maximum Relative Frequency Surpasses the Stand-Ard Afla-Toxin Limit	Relative Abundance Surpasses Iranian Veterinary Org's AFM1 Limit	
Milk truck	57	17.73±18.75	12.75	22.71	0	113.78	1.7	0	0.94
Traditional animal husbandry	62	18.94±52.72	5.55	32.32	0	416.97	1.6	0	
Industrial animal husbandry	31	21.47±26.93	11.42	31.53	0	83.68	0	0	
Milk collection center	39	16.40±18.66	10.35	22.45	0	75.63	0	0	
Total	189	18.45±34.61	13.47	23.43	0	416.97	1.06	0	

Table 3. Absolute and relative contamination frequency of North Khorasan cities to AFM1

Location	Type of Milk	Number of Samples	%		
			Iran's Standard Limit- (100 ng/L)	Iranian Veterinary Organization Limit (500 ng/L)	Union Europe Limit (50 (ng/L)
Esfarayen	Raw	26	3.8	0	3.8
Bojnurd	Raw	27	0	0	0
Dairy companies	Pasteurized	70	5.7	0	11.6
Jajarm	Raw	26	3.8	0	7.96
Raz	Raw	20	0	0	0
Shirvan	Raw	22	0	0	9
Faroj	Raw	28	0	0	17.85
Garmeh	Raw	20	0	0	5
Mane and Semelghan	Raw	20	0	0	5

regulatory framework to safeguard public health [8-10, 14-21]. Iran Veterinary Organization is responsible for monitoring the health of raw milk and raw livestock products in Iran and has established a limit of 500 ng/L for AFM1 [22]. This has led to varying risk assessments by researchers. Similar double standards are also observed in other countries [23]. Accordingly, public health and economic factors have influenced the adoption of different standards, with major agricultural producers tending to have more lenient laws and importers having stricter ones. This highlights the need for global harmonization of standards to ensure the safety and quality of food products and to protect public health.

Switzerland has implemented the strictest restrictions on aflatoxin B1 by banning the use of peanut meal in livestock diets. However, this resolution may not be practical in countries that are major producers of such products [23]. These differences have led some countries to establish different limits for AFM1 based on age groups, such as children and adults, to prevent adverse effects (Table 5) [23]. This approach ensures that milk intended for children has lower AFM1 levels than that intended for adults. For instance, Brazil, which has set a limit of 500 ng/L for raw milk, has established a stricter limit of 10 ng/L for infant milk, which is more stringent than some European countries [23]. This approach can help minimize economic losses and protect the rights of

Table 4. Absolute and relative frequency of AFM1 level in types of milk in North Khorasan Province

Types of Milk	AFM1 Level	No. (%)
Raw milk	0-50	177(93.65)
	51-100	10(5.29)
	101-500	2(1.05)
	>500	0(0)
Pasteurized and sterilized milk	0-50	61(87.14)
	51-100	4(5.7)
	101-500	4(5.7)
	>500	0(0)

Table 5. AFM1 limit standards in raw milk in different countries

Country	Adults (ng/L)	Children (ng/L)
Argentina	500	100
Australia	50	10
Brazil	500	10
France	200	
Germany	50	10
Italy	50	50
Holland	50	50
Switzerland	50	10
FDA	500	

consumers, particularly children who are more vulnerable to the harmful effects of aflatoxins. However, it also highlights the need for global harmonization of standards to ensure consistent protection of public health.

This study did not find a statistically significant difference in the mean AFM1 concentrations between raw and pasteurized milk samples collected from various cities in North Khorasan Province, Iran. Therefore, the overall mean AFM1 concentration in raw and pasteurized milk across the province can be considered a benchmark for the year 2022. This level of contamination is lower than that reported in some other studies conducted in different parts of the country. The study found that the relative abundance of AFM1 was 1.05% in raw milk and 5.7% in pasteurized milk, which exceeds the permissible limit of the national standard of Iran. Nevertheless, none of the samples in either raw or pasteurized milk exceeded the permissible limit set by the country's veterinary organization (Table 4). Overall, the AFM1 contamination in raw and pasteurized milk in the province was considered to be in good condition and did not pose a significant health risk to the population, particularly adults. In a similar study, consumption of pasteurized milk and dairy products did not pose a risk of liver cancer to children and adults in Tehran City, Iran [18]. The study found that if the EU's standard limit of 50 ng/L is applied, 6.34% of raw milk, and 11.04% of pasteurized and sterilized milk in the province exceed the limit, which is concerning given that children and elderly people are more susceptible to the harmful effects of AFM1 (Table 4). In a similar study conducted in Shahrood City, Iran, the level of AFM1 in school milk was reported to be 100% lower than the Iranian standard limit and 15.4% higher than the EU limit [8]. In another study, Sotoudeh et al. investigated the risk of carcinogenesis associated with AFM1 in milk. They calculated the AFM1 carcinogenic risk index, called the hazard index, in pasteurized milk samples from Kerman City and Rafsanjan City, Iran, which had an average AFM1 concentration of 30.9 ng/L. The results showed that milk consumption did not pose a health risk to adults, although the risk index increased for children, it did not pose a significant risk [5]. The study found that the average concentration of AFM1 in pasteurized milk was 21.54 ng/L, which is lower than the average concentration reported in the cities of Kerman and Rafsanjan, Iran, and is considered to be safe. In contrast, a study conducted in Qazvin City, Iran, reported average concentrations of 734 ng/L in raw milk and 268 ng/L in pasteurized milk, which are higher than the permissible limits of common standards. Therefore, a risk assessment for minors should be conducted to determine the potential health risks associated with these high levels of AFM1 [24]. To ensure consistency across the country, it is recommended to establish a single standard limit for children based on the level of AFM1 contamination in milk. Additionally, milk with lower levels of AFM1 contamination should be used to produce milk powder, cerlac, school milk, and other similar products. An analysis of research conducted in Iran over the past 20 years (2002 to 2022) indicates that the average concentration of AFM1 in raw milk samples has gradually decreased [5-28]. Over the past 20 years (2002 to 2022), the average concentration of AFM1 in raw milk samples has gradually decreased in Iran. The average concentrations in different regions were as follows: Tehran=207

ng/L, Ahvaz=155.91 ng/L, Gilan=123.2 ng/L, Mashhad=116 ng/L, Shiraz=112 ng/L, Babol=102 ng/L, Gorgan=76 ng/L, Isfahan=65 ng/L, Mazandaran=63.8 ng/L, Semnan=55.1 ng/L, Sanandaj=50.3 ng/L, Qazvin=38.8 ng/L, Kerman and Rafsanjan=30.9 ng/L, Yazd=22.07 ng/L, and North Khorasan=19.2 ng/L [5, 7, 9, 14, 18, 19, 25-28]. The high prevalence of aflatoxin in Gilan City, Iran, is attributed to the region's high rainfall and humidity [9], while in Ahvaz City, the storage of animal feed is likely the cause of the high aflatoxin prevalence due to the growth of fungi and the production of AFM1 [14]. However, since 2001, the overall trend of decreasing average AFM1 concentrations can be attributed to the control measures implemented by relevant organizations. The Iranian Veterinary Organization, which is responsible for monitoring the health of raw milk, has implemented a systematic program for the control, supervision, and monitoring of raw milk during the past two decades.

Similarly, through subsidiary organizations, the university of medical sciences is responsible for monitoring dairy factories and has implemented similar measures. The combined efforts of these measures have had a positive effect in reducing the concentration of AFM1 in raw milk over the years. Therefore, it is necessary to continue with group cooperation to control and monitor milk and dairy products, especially in areas prone to fungal growth and AFM1 production due to weather and climate conditions.

Conclusion

The levels of AFM1 contamination in both raw and pasteurized/sterilized milk samples from North Khorasan Province, Iran, were within the permissible limits for public consumption. However, given that children are more sensitive to aflatoxins, there is a need for a single standard to ensure the safety of all consumers.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of North Khorasan University of Medical Sciences (Code: IR.NKUMS.REC.1400.162).

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Authors' contributions

Conceptualization, methodology, data analysis, and writing of the original draft: Mitra Salehi and Pezhman Bahari; Data collection and interpretation: Mahyar Sharifan and Touhid Valizadeh; Review, editing and final approval: Hamidreza Shoraka and Akbar Solati.

Conflict of interest

The authors declared no conflict of interest.

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