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Evaluation of pH, Microbial Contamination, and Aflatoxin M1 Levels in Raw Milk: A Health Risk Assessment

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ABSTRACT

Background: This research aimed to assess pH, microbial load, and aflatoxin (AF) M_1 levels in milk from cattle dairy farms and milk distribution and collection centers in Ilam province. **Methods:** In this study, a total of 84 raw milk samples from Ilam province, collected between April and June, were analyzed for total bacterial count (TBC), pH, and AFM_1 using high-performance liquid chromatography. Daily AFM_1 intake was calculated to assess health risks based on milk consumption, using different risk assessment indices. **Results:** The highest microbial load was found in farms Location 5 (7.84 log CFU/ml) and the lowest average microbial load was recorded in farms Location 4 (5.92 log CFU/ml), while collection centers showed an average microbial count of 5.48 log CFU/ml, also linked to Location 4. AFM_1 was found in 84 samples: 65 had levels below 20 ng/g, 17 ranged from 20 to 90 ng/g, and 2 exceeded 150 ng/g, surpassing safety standards. The analysis indicates that young children consuming milk may face health risks from AFM_1, notably liver cancer. The increased incidence of elevated liver cancer risk (LCR) and associated hazard index (HI) values in children under the age of seven necessitate the implementation of more stringent regulations regarding AFM_1 contamination in dairy products, specifically targeting this demographic. **Conclusion:** Although the risk of liver cancer declines with age, it is imperative to maintain vigilant monitoring of cumulative exposure to AFM_1. This is particularly important for older adults, who may represent a vulnerable population with heightened susceptibility to AFM_1-related health complications. Ensuring rigorous surveillance and enforcement of safety standards for aflatoxins levels in milk products is essential for protecting these at-risk groups from potential adverse health outcomes.

Introduction

Food security is crucial for ensuring that the population has access to a sufficient quantity and quality of food (Augustin *et al.*, 2016). In Iran, milk is provided to school students to combat malnutrition among children. Milk is a key food for human nutrition, especially for children, as it contains essential macronutrients and micronutrients that meet the nutritional needs of all age groups. It is also a significant source of high-quality proteins that supply essential amino acids

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(Hashemi, 2016). Although milk offers various health benefits, it is susceptible to contamination by mycotoxins, rendering it unsafe for consumption and potentially leading to health complications (Kortei *et al.*, 2022). The Food and Agricultural Organization (FAO) report states that the mean value of per capita milk consumption globally is approximately 100 kg/year, but this can vary significantly between countries, ranging from 10 to 300 kg/year (Rahmani *et al.*, 2018).

Approximately 25% of food products are contaminated yearly with mycotoxins, known as secondary toxic byproducts of fungi. Aflatoxins (AFs) comprise about 90% of around 300 different mycotoxins (Smith et al., 2020). AF is a type of mycotoxin produced by *Aspergillus* fungi, including *A. nomius*, *A. parasiticus*, and *A. flavus* (Mahmoudi and Norian, 2015). AFB_1 can contaminate human food such as cereals, corn, rice, oilseeds, tree nuts, and spices (Assaf *et al.*, 2020, Marchese *et al.*, 2018), as well as animal feed such as peanut meal, maize, and cottonseed meal (Kamkar *et al.*, 2011). When consumed by dairy animals, it is absorbed into their gastrointestinal tracts and metabolized in their livers to produce AFM_1, which is then secreted into the milk of lactating mammals, thus contaminating milk and dairy products (Marchese *et al.*, 2018).

The presence of this contamination may lead to adverse effects including liver toxicity, mutagenicity, genotoxicity, teratogenicity, carcinogenicity, neurotoxicity, cytotoxicity, immunosuppression, and neoplastic effects (De Roma *et al.*, 2017, Fakhri *et al.*, 2019, Hashemi, 2016, Pour *et al.*, 2020). As a result, in 2002, the International Agency for Research on Cancer (IARC) classified AFM_1 as a Group 1 compound, which means it is carcinogenic to humans (World Health Organization and International Agency for Research on Cancer, 2002). AFM_1 is found worldwide, particularly in tropical and subtropical climates with varying temperatures, rainfall, and humidity (Fakhri *et al.*, 2019). Contamination can occur during the processing and storage of milk and its products. Moreover, AFM_1 is resistant to heat, pasteurization, autoclaving, and other food processing methods. Therefore, to ensure high-quality milk, feeds must be free from AFB_1 contamination (Omar, 2016, Sadeghi *et al.*, 2009). Due to the high toxicity and heat stability of AFM_1, coupled with the significant consumption of milk and dairy products, various countries have established maximum levels (ML) of AFM_1 in milk, which vary widely based on their economic status and development (Kortei *et al.*, 2022, Mollayusefian *et al.*, 2021). The European Union (E.U.) and the U.S. Food and Drug Administration (FDA) have set the ML for AFM1 in liquid milk at 50 and 500 ng/L, respectively (Hassan *et al.*, 2018). Hence, it is crucial to recognize that AFM_1 contamination in milk of dairy cattle presents a significant risk and should not be overlooked. Ongoing global research is being conducted on the presence of AFM_1 in raw milk, highlighting the seriousness of this issue (Ketney *et al.*, 2017). It is essential to continue investigating the presence of AFM_1 in raw milk to gather data on human exposure and to assess the potential long-term health risks associated with consuming low levels of AFM_1 in milk (Ketney *et al.*, 2017).

One of the most effective ways to determine the likelihood and severity of liver cancer risk (LCR) is quantitative risk assessment for milk and dairy products contaminated with AFM_1 (Tsakiris *et al.*, 2013). This research aimed to assess pH, microbial load, and AFM_1 levels in milk from cattle dairy farms and distribution and collection centers in Ilam province, Iran. Furthermore, the health risks of AFM_1 across various age groups were evaluated by calculating cancer risk and considering factors such as estimated daily intake (EDI), hazard index (HI), margin of exposure (MoE), and LCR.

Materials and Methods

Description of the study site

Ilam province, situated in the western region of Iran, is inhabited by approximately 557,599 residents and encompasses a total area of 20133 square kilometers. Its geographical coordinates range between 45°24' and 48°10' longitude, and 31°58' and 34°15' latitude. The province was

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geographically partitioned into six distinct regions, delineated based on the density of cattle farms and the location of milk collection and distribution centers, as depicted in **Figure 1**.

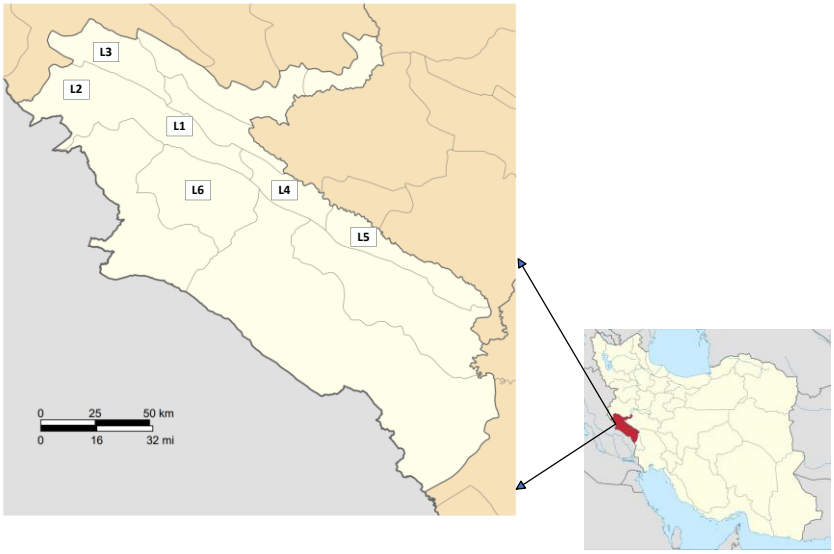

Figure 1. Sampling locations in Ilam province are as follows: L1 in Ilam County, L2 in Chavar County, L3 in Ivan County, L4 in Badreh County, L5 in Darehshahr County, and L6 in Malekshahi County.

Data collection

This study analyzed 84 raw milk samples, comprising 47 from dairy farms and 37 from collection and distribution centers in the province. Sampling was conducted randomly from April to June 2019, with each center selected based on specific criteria. Before sampling, milk temperature was measured, and 100 milliliters were collected into two separate falcon tubes—one for microbiological tests and one for chemical tests. The samples were promptly transported to the laboratory under cold conditions (4±1 ^{0}C) for further examination.

Determination of the microbiological load

Total bacterial count (TBC) was performed to assess the microbial load in raw milk samples, following the guidelines outlined by the Iranian National Standardization Organization (INSO, n: 5272-2) (Iranian National Standardization Organization, 2008).

Determination of pH

The pH meter was calibrated using pH 4 and pH 7 buffer solutions prior to measurements. A small amount of milk was placed into a 30- to 50-milliliter flask and stirred using an electric mixer until it was completely smooth (Gemechu and Amene, 2016).

AFM_1 analysis

Raw milk samples were collected from all dairy farms and dairies in Ilam province. The samples were stored in 100 ml Falcon tubes and frozen at -18 ^{0}C until AFM1 determination test was conducted. To measure AFM1 levels in milk, the samples were processed according to the guidelines outlined in reference method a-5925 of the ISIRI (Iranian National Standardization Organization, 2020). Initially, 60 ml of milk was heated to 37 °C, after which the fat was separated from milk through centrifugation, and the upper layer was discarded. Subsequently, an immunoaffinity column was used to purify milk samples. It should be noted that homogenization prevents fat separation during centrifugation, rendering this statement inaccurate.

The milk was centrifuged at 2000 ×g to separate fat, and the upper layer was discarded. Subsequently, 10 ml of phosphate-buffered saline solution was added to immunoaffinity column. After passing through two stages of filter paper, 20 ml of skim milk was passed through the column.

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The sample container was washed twice with grade 3 water, and wash water was passed through the column. The column was then washed with 10 ml of grade 3 water. Then, 2500 µl of acetonitrile was passed through the column at a flow rate of 2-3 ml/min, collected in a vial, and dried in a water bath at a temperature of 50-40 ^{0}C. Subsequently, 1 ml of mobile phase was added to the vial and mixed for the second time with a vortex mixer (Labnet, Tehran, Iran). The column was washed with 20 ml of phosphate saline buffer, and finally, 200 µl of this solution was injected into the HPLC device. The resulting peaks from the inhibition test were compared with standard peaks to identify and quantify the contamination, as determined by the calibration curve (**Figure 2**).

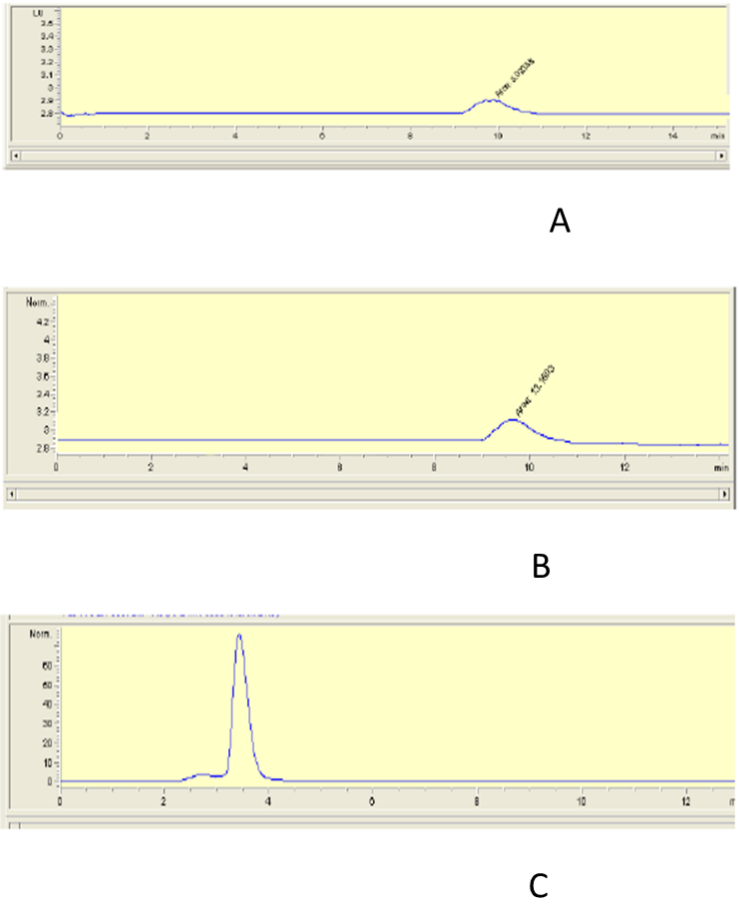

Figure 2. HPLC chromatograms of AFM1 standard solution (A: 100 ng/g, B: 100 ng/g<, C: 100 ng/g>).

Risk assessment of exposure to AFM$_1$

Estimation of exposure: To assess the potential risks of daily milk consumption and exposure to AFM1, the first step was to estimate the daily intake of AFM$_1$ in ng/day.kg of body weight. This calculation was performed using the following equation (Hooshfar *et al.*, 2020, Sharma *et al.*, 2020):

$$EDI=\frac{C\times I\times E}{B.W} \qquad \textbf{Equation 1}$$

In the provided equation, C represents the concentration of the contaminant (AFM$_1$) in the milk (ng/l), I stands for daily milk intake (L/d), E denotes the exposure duration per day, and B.W represents the average weight of individuals in the test population (kilograms). According to the report from the Agriculture Ministry of Iran, the average daily milk intake for the general population was approximately 0.25 L (Moghaddam *et al.*, 2019, Nejad *et al.*, 2020, Sharma *et al.*, 2020). BW represents the mean body weight, which was assumed to be 15, 45.2, 78.7, and 74.5

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kg for males under 7, aged 7–16, aged 16–65, and over 65 years old, respectively. For females, these values were assumed to be 15, 43.9, 65.5, and 66.3 kg, respectively (United States Environmental Protection Agency, 2011).

MoE characterization for AFM_1: Genotoxic and carcinogenic substances like AF are appropriately evaluated for risk using the MoE method. These three indicators were determined based on the average daily intake of AFM_1 and the average milk consumption within the community. The assessment of the risk of AFM-induced liver cancer involved multiplying the probability of cancer by the average AFM_1 exposure (Benford *et al.*, 2010).

When calculating the MoE for average exposures to AFM, the Benchmark Dose (BMD) was utilized as the dose showing the least measurable response (570 ng/d.kg bw, representing AFM potency for hepatocellular carcinoma (HCC) based on a 2-year study in male Fischer rats) (Serraino *et al.*, 2019, Udovicki *et al.*, 2019). This value was determined by dividing the reference value by the EDI in consumers (European Food Safety Authority, 2005, Udovicki *et al.*, 2019). Any value above 10,000 was regarded as a low-level public health concern.

$$\mathrm{MoE} = \frac{570\frac{ng}{kg}bw/day}{EDI\frac{ng}{kg}bw/day} \qquad \textbf{Equation 2}$$

Health index (HI) of AFM_1: The HI was determined using **equation (3)** to ensure the safety of milk about AFM_1. In this equation, the EDI represents the average daily intake of AFM_1 through milk consumption, and RFD is the reference dose derived from the TD50 of AFM_1 (10.38 µg/kg bw/day). The TD50 is the amount of AFM_1 that causes tumors in half of the laboratory animals. The specified TD50 value was divided by an uncertainty factor of 50,000 to obtain the RFD for AFM_1 in humans, resulting in a risk of 1:100,000. Considering this process, the value of RFD was established at 0.2 ng/kg bw/day. If the HI value is equal to or less than 1, it will be considered an acceptable risk. Conversely, if the HI value exceeds 1, it will be regarded as an unacceptable risk, indicating a potential increase in LCR (Bahrami *et al.*, 2016, Hooshfar *et al.*, 2020).

$$HI = \frac{EDI\frac{ng}{kg}bw/day}{RFD\frac{ng}{kg}bw/day} \qquad \textbf{Equation 3}$$

Estimated LCR: People infected with hepatitis B virus (HBV) seem to be at a greater risk of developing HCC as a result of exposure to AFs. This study examined the combined impact of exposure to AFM_1 and HBV on the development of HCC. It used a specific formula to calculate the estimated risk of HCC (cancer cases per year per 100,000 people) due to AFM_1 exposure in the community.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) provided the LCR assessment method related to AFM1 (FAO/WHO, 2017). JECFA indicated a potential LCR exposure of 1 ng AFB1/kg bw/day in 100,000 individuals. Consequently, the estimated potential LCR attributed to AFM1 was 0.049 (95% upper bound) additional cancer cases per 100,000 for HBsAg-negative individuals and 0.562 (95% upper bound) for HBsAg-positive individuals.

AFM_1 has a lower carcinogenic potency compared to AFB_1, even in sensitive species such as rainbow trout and Fischer rats, with a potency that is one-tenth of AFB_1. The carcinogenic potency of AFB_1 for HBsAg- and HBsAg+ has been documented as 0.0049 and 0.0562, respectively (FAO/WHO, 2017, Hooshfar *et al.*, 2020). The prevalence of HBsAg+ in Iran, according to the report of Iran's Center for Disease Control and Prevention, is 1.5%. Therefore, the percentage of the population (Pop) related to HBsAg+ and HBsAg- is 0.015 and 0.985, respectively (Hooshfar *et al.*, 2020, Moghaddam *et al.*, 2019). **Equations (4)** and **(5)** were used, based on the information mentioned above, to calculate the cancer potency (CP) and cancer risk (CR) generated by AFM_1. As per the US Environmental Protection Agency guidelines, a carcinogenic risk lower than 10^{-4} is deemed acceptable, while a risk higher than 10^{-4} is considered to be carcinogenic (Environmental Protection Agency, 2001).

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$CP = (PHBAg^{+} \times \%PopHBsAg^{+}) + (PHBsAg^{-} \times \%PopHBsAg^{-})$ **Equation 4**

$LCR = CP \times EDI$ **Equation 5**

Data analysis

The statistical analysis was conducted using IBM SPSS version 20 software (SPSS Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA) test was used to compare mean scores between cities, distribution centers, and dairy farms. The average microbial load and pH were also compared using an independent t-test. Both tests were performed at a significance level of $\alpha = 0.05$ and a confidence level of 95% CI.

Results

TBC

The examination of TBC in raw milk from dairy farms in Ilam province revealed that L5 had the highest microbial load (log CFU/ml 7.84), while the lowest was observed at L4 (log CFU/ml 5.91) (**Figure 3. A**). A statistically significant difference in microbial load was found between these locations ($P<0.05$).

Similarly, when investigating the TBC in raw milk at the collection and distribution centers of Ilam province, it was found that the lowest TBC was associated with L1 (5.25 log CFU/ml) (**Figure 3. B**), and this difference was also statistically significant ($P<0.05$). The mean TBCs in samples collected from L1, L4, and L5 were 5.25, 8.08, and 8.57 log10 CFU/ml, respectively. A high TBC was detected in raw cattle milk sample collected at L4, while low counts were detected in milk sample collected at L1. Except for raw milk collection centers in L1, the other areas were not up to standard.

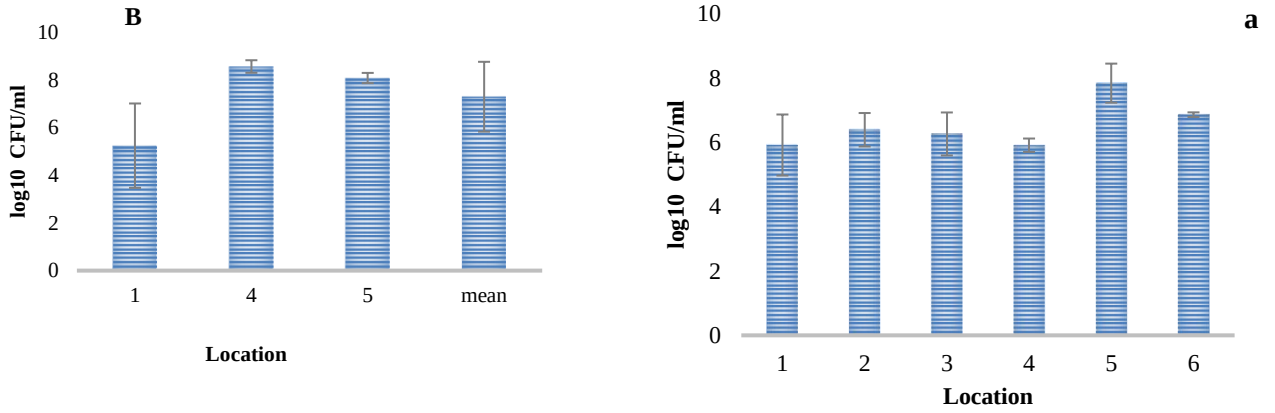

Figure 3. TBC in raw milk of dairy farms (A) and collection and distribution centers (B) in Ilam province (mean±SD).

pH

The average pH levels in cattle dairy farms and collection and distribution centers in Ilam province are provided in **Table 1**. The results indicated that the lowest pH value in raw milk collected from dairy farms in Ilam province was associated with raw milk from cattle farms in L5 ($P<0.05$). It is important to emphasize that only three milk collection centers were located in areas 1, 4, and 5, from which samples were obtained. The highest quantity was associated with L4.

Table 1. Mean pH in dairy farms and collection and distribution centers in Ilam province (mean ±SD).

Sampling Location	Dairy farm	Collection Center
1	6.64±0.10	6.28±0.33
2	6.67±0.21	-
3	6.70±0.10	-
4	6.5±0.014	6.32±0.035
5	6.41±0.169	6.305±0.09
6	6.53±0.014	-

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Evaluation of AFM_1

This study investigated the level of AFM_1 in 84 raw milk samples collected from cattle farms and collection centers in Ilam province. According to the findings (**Table 2**, **Figure 4**), 65 samples showed AFM_1 contamination levels below 20 ng/l (the detection limit of the device). A total of 17 samples exhibited contamination levels ranging from 20-90 ng/l, while two samples surpassed 100 ng/l, exceeding Iran's permissible limit. According to the European Union, the concentration of AFM1 exceeded the permissible limit of 50 ng/l in seven samples.

AF mean levels in two cohorts of dairy cattle farms and collection centers were compared. As indicated in **Figure 3**, the farm (25.96 ng/l) exhibited a higher AFM_1 level than the centers (15.14 ng/l). Nevertheless, both mean levels remained below the permissible limit.

Table 2. Occurrence of AFM1 in raw cattle milk in Ilam province, Iran.

Cohort	Sample(n)	Distribution of samples n (ng/l)				
		<20	20-50	50-100	100-200	>500
Dairy farm	47	38	5	3	1	0
Collection and distribution centers	37	27	7	2	0	1
Total (N)	84	65	12	5	1	1

The Iran National Standards Organization (INSO) has set a maximum allowable level of 100 ng/l for AFM1 in milk. The European Union has established a limit of 50 ng/l for AFM1 in milk, while the Food and Drug Administration has set the maximum permissible level for AFM1 in milk at 500 ng/l.

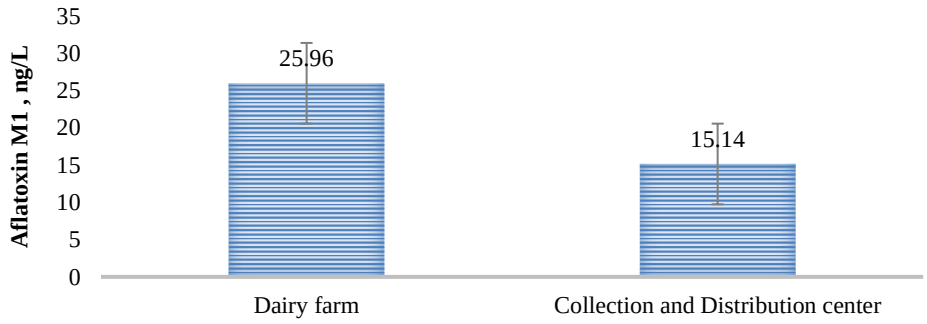

Figure 4. AFM_1 concentration results in dairy farms and collection and distribution centers in Ilam province (mean±SD).

Health risk assessment of AFM_1

The EDI of AFM_1 through milk consumption presented in **Table 3** is supported by health hazard indices such as HI, MoE, and LCR for different ages and sexes. EDI of total AFM_1 in raw cow milk samples from Ilam province were 0.36, 0.12, 0.07 , 0.07, and 0.06 ng/kg bw/day for male age groups including <7, 7-16, 16-6 ,and >65 years, respectively, and for this, female age groups had respective daily intakes of 0.36, 0.12, 0.08, and 0.08 ng/kg bw/day. The highest EDI for males and females in age groups below 7 years was 0.36 ng/kg bw/d, since younger age groups consume more milk per body weight compared to older age groups. The data on EDI showed a remarkable decrease with age, and values for age groups 16-65 and >65 years were quite low, ranging from 0.07 to 0.08 ng/kg bw/day.

The latest findings of the study revealed that among males, the HI rates for milk consumption were 1.79, 0.59, 0.34, and 0.36 in the age groups <7, 7–16, 16–65, and >65 years, respectively. For

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females in the corresponding age groups, the HI rates were 1.79, 0.61, 0.41, and 0.4, respectively. The study showed that the potential non-carcinogenic effects of AFM_1 from milk consumption in the age group >7 exceeded the allowable limit (**Table 3**). In the younger age group (<7 years), the HI was determined to be 1.79 for both males and females, raising concern for significant risk due to AFM1 exposure through milk during this developmental stage. On the contrary, the HI decreased with age, and the lowest values of 0.34-0.41 were recorded for the 16-65- and >65-year age groups, indicating a low non-carcinogenic risk in adults.

The MoE level serves as a pivotal parameter for characterizing risk, with a value below 10,000 indicating a potential health hazard. The current study findings demonstrated that the MoE value for AFM_1 in all types of milk sourced from diverse sampling locations and across all age groups of both genders was consistently recorded below 10,000 (**Table 3**). This suggests a tangible health risk associated with the consumption of AFM_1-contaminated milk. Moreover, MoE highlighted the health risks associated with the presence of AFM_1 in milk. For both males and females under 7 years, the MoE was the lowest at 1595.98, indicating a higher risk compared to older age groups. In contrast, values of MoE were considerably higher for older children (7-16 years), with up to 4808.96 in males and 4670.65 in females. For adults, 16-65 years old , it increased further, indicating lower carcinogenic risk with age. However, a slight decrease was observed in MoE values for the >65-year age group, representing marginally increased vulnerability among the elderly.

In the current study, the results of LCR calculation indicated that for all types of milk collected from various sampling locations and for all age groups of males and females, the LCR level was higher than 4-10 (**Table 3**). However, the LCR level for children under 7 years old (2.025×10^{-3}) was below 10^{-4}, indicating its carcinogenicity. The highest estimated LCR level was in males and females under 7 years at 2.025E-3, indicating a higher risk of liver cancer in this age group. The LCR decreased with age, indicating reduced exposure and risk in the 16-65 age group. However, in the >65 years group, the LCR was still lower compared to other age groups, but considerably indicated the long-term effects of chronic exposure to AFM_1.

Table 3. The EDI and HI, MoE, and LCR of AFM_1 via milk consumption based on various age groups for males and females

Sex and age groups(year)	EDI (ng/kg bw/day)	MoE	HI	LCR
Male				
<7	0.36	1595.98	1.79	2.025E-3
7-16	0.12	4808.96	0.59	6.72E-4
16-65	0.07	8373.12	0.34	3.86E-4
65<	0.07	7947.55	0.36	4.07E-4
Female				
<7	0.36	1595.98	1.79	2.025E-3
7-16	0.12	4670.65	0.61	6.91E-4
16-65	0.08	1393.75	0.41	4.64E-4
65<	0.08	7053.85	0.40	4.58E-4

EDI: *Estimated daily intake;* ***MoE:*** *Margin of exposure;* ***HI:*** *Health index;* ***LCR:*** *Liver cancer risk*

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Discussion

Evaluation of TBC, pH, and AFM_1

Iran Veterinary Organization (IVO) has established standard limits for TBC in raw milk, which have been categorized into four groups: excellent (≤4.48), first-grade (4.48–5), second-grade (5–5.70), and third-grade (5.70–6) log10 CFU/ml. The results showed that TBC levels in milk from all regions exceeded the standard. High TBCs can result from insufficient milk cooling, inadequate udder preparation methods, and unclean milking equipment.

The findings in L3 and L4 showed higher TBC levels compared to those reported by Arefeh *et al*., who recorded a TBC of 5.25±0.13 in Kerman, Iran (Arefeh *et al.*, 2021). Its mean value (7.30) was mostly based on values reported by Kazeminia *et al.*, who found 6.41±1.22 in milk sampled from milk collection centers in Qazvin, Iran (Kazeminia *et al.*, 2023). Elevated TBC levels may be indicative of a diseased udder, improper milk handling practices, or unfavorable storage temperatures (Nasir and Gemede, 2024). The variation in the collected samples could be attributed to unhygienic handling of milking apparatus. This suggests that equipment management during milking process could significantly affect the variability in the samples.

TBC levels observed in this study exceeded the acceptable thresholds established by both national and international regulatory standards. These limits are set to ensure food safety and public health, as elevated TBC levels indicate potential microbial contamination, which can compromise product quality and pose significant health risks to consumers. Compliance with these standards is crucial for minimizing the likelihood of foodborne illnesses and ensuring the microbiological safety of consumable products.

Analysis of the samples revealed significant differences in pH levels and TBC growth across various sampling locations (P<0.05). In L5, where the pH was lower than in other areas, the number of microorganisms was higher. This is in agreement with the previous research that obtained an average pH value of 6.30±0.41 (Kazeminia *et al.*, 2023). Furthermore, Moosavi *et al.* in Tabriz, Iran, reported that the pH of most samples ranged from 5.00 to 7.00, with an average of 6.75 (Moosavi *et al.*, 2018). This increase can be attributed to the growth of psychotropic microorganisms at lower temperatures and the production of acidic metabolites.

A review study analyzed 11,370 samples of AFM_1 from various types of milk in Iran. The distribution of samples was as follows: 21.7% from dairy factories and milk collection centers (location 1), 48.8% from livestock farms (location 2), 26.8% from distribution centers and the milk market (location 3), and 2.7% from unspecified locations (location 4). The average AFM_1 level was 77±159 ng/kg (ND-1137 ng/kg), surpassing the EU limit of 50 ng/kg, but remaining under the INSO threshold of 100 ng/kg (Massahi *et al.*, 2023). Furthermore, the study revealed that livestock farms had the highest AFM_1 levels (90±209 ng/kg) among milk sampling locations, with raw milk showing a significant correlation (84±84 ng/kg) (Massahi *et al.*, 2023), consistent with the current findings.

Hasninia *et al.* also found that the average total AFM_1 of raw milk was 31.2±1.7 ng/l (Hasninia *et al.*, 2022). The variation in AFM_1 levels was primarily affected by type of milk, timing of sampling, and presence of AFB_1 in the feed given to lactating animals. Furthermore, differences in AFB_1 levels can arise from various factors, including conditions under which animal feed is stored, such as ventilation, temperature, humidity, storage duration, and type of feed itself; therefore, each of these factors can significantly affect the growth of *Aspergillus* fungi that produce AFB_1. AF-producing fungi thrive in conducive environments characterized by humidity levels exceeding 15%, minimum temperatures of 25 °C, sufficient oxygen, and particularly warm and humid climates affecting cereals and animal feed (Berghofer *et al.*, 2003, Ghaneian *et al.*, 2016, Ranjbar *et al.*, 2010, Sales and Yoshizawa, 2005). Iran's diverse geography leads to a variety of weather conditions, contributing to different environments that can promote the growth of AF-producing fungi across different regions of the

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country.

Health risk assessment of AFM_1

AFM_1 is a metabolite of AFB1 that contains carcinogenic properties; therefore, its presence in milk is dangerous for the health of consumers, especially in liver cancer. Health risk indicators were analyzed across various populations, offering insights into age- and sex-related changes. The results of similar studies align with the present study findings on EDI. Massahi *et al.* reported EDI values of 0.94, 0.42, 0.24, and 0.25 ng/kg bw/day for age groups <7, 7-16, 16-65, and >65 years, respectively, but for females, the corresponding values were 1.26, 0.43, 0.29, and 0.28 ng/kg bw/day, respectively (Massahi *et al.*, 2023). Hasninia *et al.* also found that EDI values from milk consumption in summer for males were 0.943, 0.542, and 0 for the age groups 7-16, 16-65, and over 65 years, respectively, while for females in the same age groups, the values were 0.971, 0.651, and 0.643 ng/kg bw/day, respectively (Hasninia *et al.*, 2022). Based on the findings of the study, during winter, the estimated exposure range for males in the specified age groups was 0–0.314, 0–0.181, and 0–0.191 ng/kg bw/day, respectively. For females in the same age groups, the exposure range was 0–0.324, 0–0.217, and 0–0.214 ng/kg bw/day, respectively (Hasninia *et al.*, 2022).

Pardakhti *et al.* reported that the EDI of AFM_1 from milk consumption in various cities was as follows: Tehran (0.057 ng/kg bw/day), Mashhad (0.063 ng/kg bw/day), Ahwaz (0.118 ng/kg bw/day), Babol (0.289 ng/kg bw/day), Esfahan (0.069 ng/kg bw/day), Kermanshah (3.319 ng/kg bw/day), Miandoab (0.007 ng/kg bw/day), Hamedan (0.036 ng/kg bw/day), and Urmia (0.210 ng/kg bw/day) (Pardakhti and Maleki, 2019). Variations in the EDI of AFM_1 between studies can be attributed to multiple factors, such as the concentration of AFM_1, daily milk intake (DMI), consumer age group, and body weight (BW), as well as the type, quality, and storage conditions of dairy cattle feed (Massahi *et al.*, 2023).

In a related study, HI values for males in Iran consuming milk, across the age groups of under 7 years, 7 to 16 years, 16 to 65 years, and over 65 years were reported to be 4.7, 2.1, 1.2, and 1.3, respectively, while for females in the same age groups, HI values were 6.3, 2.1, 1.4, and 1.4, respectively (Massahi *et al.*, 2023), which was higher than the values observed in the present study. The variation in HI across different age groups is attributed to differences in EDI, the possible causes of which have been discussed earlier. According to the findings, the potential HI for non-carcinogenic effects associated with AFM_1 exposure due to milk consumption in Iran exceeded the permissible limit, particularly for children and adolescents. Since an HI value greater than 1 indicates an increased risk of liver cancer, immediate action is required to mitigate AFB_1 contamination in dairy cattle feed, especially during the summer season. Key interventions should focus on disrupting the growth conditions of *Aspergillus* species found in animal feed storage areas (Berghofer *et al.*, 2003, Ghaneian *et al.*, 2016, Ranjbar *et al.*, 2010, Sales and Yoshizawa, 2005). A review by Rahmani *et al.* reported that HI values for AFM_1 exposure through raw milk consumption in adults from Iran, Turkey, Syria, Palestine, Lebanon, and Egypt were 0.26, 0.47, 0.52, 0.34, 0.23, and 0.18, respectively (Rahmani *et al.*, 2018). As mentioned earlier, variations in parameters affecting the EDI of AFM_1, such as BW, DMI, and AFM_1 concentration in milk, likely explain the differences between the findings of this study and previous research.

According to EFSA Scientific Committee guidelines, MoE based on a BMD Lower Confidence Limit (BMDL) of 10,000 or more from animal studies is not considered a public health concern and is deemed a low priority for risk management (European Food Safety Authority, 2005). However, the present study results for raw milk showed that MoE values for AFM_1 exposure among consumers were below 10,000, indicating a health concern related to AFM_1 exposure. A study by Udoveicki *et al.* in Serbia and Greece assessed AFM intake in milk and yogurt, reporting MoE values ranging from 213.2 to 460.4 for Serbia and

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1142.3 to 1628.6 for Greece (Udovicki *et al.*, 2019). These findings, along with those of Hasninia and Massahi show MoE levels below 10,000 (Hasninia *et al.*, 2022, Massahi *et al.*, 2023) and Mokhtarib *et al.* with an MoE range of 1892.9 to 3921.6 (Mokhtari *et al.*, 2022), align with the current study results.

Similar studies have reported the LCR associated with AFM_1 exposure through milk consumption in urban areas, with calculated risk values ranging from 0.0008 to 0.0017, which corresponds to the second sampling series (traditional milk) and the first sampling series (pasteurized milk), respectively (Mokhtari *et al.*, 2022). These results represent the estimated probability of additional liver cancer cases per year per million individuals. Based on these findings, the overall cancer risk from AFM_1 exposure to milk appears to be very low, and there is no significant public health concern in this regard. In a study conducted by Hooshfar *et al*. in Iran (Hooshfar *et al.*, 2020), the risk assessment of primary LCR due to AFM_1 in milk revealed that cancer risk, based on a more realistic scenario accounting for average AFM_1 exposure and milk consumption, was calculated at 0.0001 additional cancer cases per 100,000 person-years. This finding aligns with the present study, which ranged from 0.0007 to 0.0016 additional cases per million person-years (Hooshfar *et al.*, 2020).

This study aimed to assess the levels of microbial contamination and the presence of aflatoxins in livestock farms throughout the province. We measured AFM_1 levels and microbial contamination in cattle milk, ensuring that all active livestock farms were included in our research. Future studies should focus on investigating the causes and sources of bacterial contamination, as well as isolating and identifying the microorganisms and bacterial strains present in milk. Moreover, it will be important to examine the levels of AFM_1 across different seasons and how seasonal variations may impact these levels.

Conclusion

This study evaluated the presence and concentration of TBC, pH, and AFM_1 in raw cattle milk in Ilam province, Iran. The analysis of TBC in raw milk samples collected from dairy farms showed that L5 exhibited the highest microbial load, with a log CFU/ml value of 7.84. In contrast, L4 had the lowest microbial load, with a recorded log CFU/ml value of 5.91. Furthermore, the lowest pH of raw milk from cattle farms was in L5, while the highest was in L3. Based on the findings, the mean AFM_1 level in raw milk cattle produced in Ilam province was dairy farms (25.96 ng/l) and collection centers (15.14 ng/l), which was below EU regulation (50 ng/l) and INSO regulations (100 ng/l). The results showed a statistically significant variation in mean levels of pH, TBC, and AFM1 between dairy farms, collection and distribution centers, and various sampling locations ($p<0.05$). These discrepancies probably result from individual lactating animals ingesting AFB1 at different levels, since AFB1 is metabolized into AFM1 and excreted into milk. The present analysis points out that in health risk assessment of AFM_1, in the case of young children, the intake of AFM_1 through milk may pose overall health risks, with a particular incidence of liver cancer. High LCR and HI values among children under 7 years suggest stricter enforcement of the regulations regarding contamination by AFM_1 in milk and its products, with special emphasis on product consumption within this age group. Although the risk decreases with age, it supports the continued vigilance in the long-term impact of cumulative exposure, particularly for the elderly, to protect vulnerable populations from AFM_1-related health risks.

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Authors' contributions

Nemati M, Khajehali E, and Rashidimehr A conducted the experiment, analyzed data, interpreted statistical analysis, and wrote the main draft of the manuscript. All authors reviewed and

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approved the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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