

AFLATOXIN M1 LEVEL IN RAW MILK AND UHT MILK CONSUMED IN KOSOVO DURING 2021- 2022

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Abstract

Aflatoxin M1 is a toxic metabolite produced by certain species of *Aspergillus*. It can be found in milk and dairy products if cows consume feed contaminated with aflatoxin B1 (AFB1). Aflatoxin M1 is a potential human carcinogen. It is toxic to the liver and can cause liver damage at high doses. The aim of this research was to determine the level of aflatoxin M1 (AFM1) in raw cow's milk in the region of Peja, one of the biggest regions of Kosovo, and in the ultra-high temperature (UHT) treated cow's milk consumed in Kosovo that was found in supermarkets which was produced in Kosovo and imported from different countries, also.

From August 2021 until April 2022, 120 raw milk samples have been collected from small dairy farms in one of the biggest regions of Kosovo, called Peja, and 40 UHT local and imported cow milk samples imported from different countries and found in supermarkets were collected and analyzed for aflatoxin M1 (AFM1), a toxic metabolite of aflatoxin B1, which may contaminate animal's feed. The contamination level of aflatoxin M1 was determined using enzyme-linked immunosorbent assay (ELISA).

A total of 75 (47%) samples were contaminated with AFM1 at levels > limit of detection (LOD) (0.002 µg/L). Among them, 33% of UHT milk samples and 52% of raw milk samples resulted positive in AFM1. Only one sample from UHT imported milk, exceeded the European Union maximum level of 0.05 µg/L, with the level of AFM1 of 0.165 µg/L. The maximum AFM1 level for raw milk was at 0.015 µg/L. There was no relevant difference between the seasons regarding to AFM1 frequency and levels for raw milk; although for the UHT

local and imported milk samples there was a difference in AFM1 level, with UHT imported milk with higher AFM1 concentration.

In conclusion, this study shows that the frequency of AFM1 contamination of raw milk in the region of Peja in Kosovo in the time interval between summer 2021 and spring 2022, was lower than the results reported on the previous studies for this region, and all the samples have been compliant with the European Union regulations for AFM1. This might show an increase in awareness of the proper storage of animal feeding stuff by farmers. As for the UHT imported milk samples, the results show that regular institutional monitoring is necessary.

Key words: Raw milk, UHT milk, ELISA, Aflatoxin M1.

1. Introduction

Milk is an important daily food source which is consumed in large quantities, especially by children and the elderly. For this reason, in addition to making sure that it contains sufficient nutritional value, we must make sure that in addition to being microbiologically pure, it is not chemically contaminated. One of the biggest concerns is its contamination with aflatoxins.

Aflatoxins are a major class of mycotoxins produced primarily by *Aspergillus* species including *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (Creppy [3]). Factors such as prolonged drought, high temperatures, substrate composition, storage time and storage conditions of crops, food

products, and feedstuff, play an important role in fungal growth and the synthesis of aflatoxins (Stack and Carlson [17]). The aflatoxins consisted of aflatoxin B₁, B₂, G₁, and G₂ may contaminate food and feed, as maize grains and other feedstuffs such as corn silage. Lactating animals that consume feed contaminated with aflatoxins excrete AFM₁ in urine and as well in the milk. Aflatoxin M₁ (AFM₁), the hydroxylated metabolite of aflatoxin B₁ (AFB₁), is a carcinogenic substance detected in milk and dairy products (Prandini *et al.*, [14]). Approximately 0.3 - 6.2% of AFB₁ is converted into metabolized AFM₁ and excreted in milk, depending on factors such as the genetics of the animals, seasonal variation, the milking process and the environmental conditions (Unusan [19]).

On this basis, this toxin must be monitored for its presence and concentration before the milk is released for consumption. Consumers from developing countries, especially from rural areas, face issues related to food security and food safety because they depend on locally produced foods (Marroquín-Cardona *et al.*, [11]). The presence of AFM₁ in milk and dairy products is an important issue, especially for developing countries (Prandini *et al.*, [14]).

The maximum level for AFM₁ in raw milk and heat-treated milk set by the European Union Commission Regulation (EC) 1881/2006 is 0.05 µg/L for AFM₁. The same regulation sets a lower maximum level of 0.025 µg/L for infant formulae and follow-on formulae, including infant milk and follow-on milk, as well as for dietary food for special medical purposes intended specifically for infants [7]. The respective Kosovo laws [13] are harmonized with the European Union Legislation with regard to maximum levels of mycotoxins.

Based on the data of a study done in 2013 in Kosovo, there was a high incidence of AFM₁ (81%) in both pasteurized and UHT milk samples. Eighty three percent (83.3%) of the pasteurized milk samples and seventy eight percent (78.7%) of the UHT milk samples contained AFM₁ (Rama *et al.*, [15]). Another study done in Kosovo during 2016 concluded that the frequency of AFM₁ contamination of raw milk in Kosovo was relatively high, and a considerable number of samples would have been non-compliant with the European Union regulations for AFM₁ (Camaj *et al.*, [2]). In terms of regional distribution of positive samples during 2009 and 2010 (Rama *et al.*, [16]), Peja was the region with the highest number of positive samples. According to the results found in the study done by Camaj *et al.*, [2], and Camaj *et al.*, [1], the region of Peja was the second on positive samples, out of five regions tested for the AFM₁ presence. In the last ten years, many studies have reported a frequency of AFB₁ in feedstuff and AFM₁ in

milk, baby milk, and infant cereals (Kos *et al.*, [9, 10]; Dimitrieska-Stojkovic *et al.*, [5]; De Rijk *et al.*, [4]; Hajnal *et al.*, [8]; Muharremi *et al.*, [12]; and Topi *et al.*, [18]).

Based on these and other data about the presence of AFM₁ in milk, especially in the region of Peja, Kosovo, the aim of this study was to analyze and evaluate the presence and concentration of AFM₁ in raw milk in the region of Peja, Kosovo, during 2021 and 2022, and the UHT milk regional and imported in Kosovo during 2022.

2. Materials and Methods

2.1 Sample collection

In total, 160 milk samples were collected, 40 samples were ultra-high treated (UHT) milk, from which 7 samples were from well-known local producers in Kosovo, and 33 samples were from different producers from abroad countries which were obtained from big food suppliers. Each sample represents a different product of different producer with different time of production. UHT milk samples were taken one day before the analysis. The rest, 120, were raw bulk tank milk samples of local farmers in the region of Peja (Figure 1), each 40 samples were of three different seasons, summer 2021, winter 2021, and spring 2022, respectively. Raw milk samples were stored at - 20 °C until the time of analyses. Sampling was done according to the European Commission 401/2006 [6].



Figure 1. Map showing the region of Peja, Kosovo

2.2 Laboratory analyses

AFM₁ was detected by enzyme-linked immunosorbent assay (ELISA) (MaxSignal Aflatoxin M₁ ELISA Kit, PerkinElmer), following the manufacturer's instructions, summarized in brief as following.

Frozen samples were brought at room temperature (20 - 25 °C). All the samples were centrifuged for 10 minutes at 4,000 x g, and then the upper layer was removed before analyses. Dilution factor was 1.

The method is based on a competitive, one step colorimetric enzyme-linked immunosorbent assay (ELISA). The analyte specific antibody has been coated to the plate wells. During analysis, sample was added along with the analyte-conjugated horseradish peroxidase (HRP). If the target analyte was present in the sample, it competes for the antibody, thereby preventing the conjugated HRP from binding to the antibody coated to the plate well. The resulting color intensity, after addition to the "TMB" substrate, had an inverse relationship to the target analyte concentration in the sample. The absorbance values were obtained using a plate reader set at 450 nm, and the level of AFM₁ was calculated using a logarithmic standard curve, and the average of the duplicates was used as results. Samples exceeding the maximum level of 0.05 µg/L were retested in duplicates.

2.3 Statistical analysis

Statistical analysis was performed using Excel. The results were grouped into five categories, as UHT regional milk, UHT imported milk, and three groups were of raw milk named according to the season of sampling, as raw milk of summer, winter, and spring season, respectively. The results obtained for the AFM₁ content were expressed as mean values with standard deviation (SD), as median, and as maximum concentration of AFM₁. Differences of AFM₁ concentration between UHT milk and the raw milk of different seasons were analyzed by applying Kruskal-Wallis ANOVA test. The differences between values were significant ($P < 0.05$).

3. Results and Discussion

The limit of detection according to the manufacturer of the testing Kit (MaxSignal Aflatoxin M₁ ELISA Kit, PerkinElmer), was 0.002 µg/L.

The results of this survey for UHT milk, both regional and imported, and for the raw milk of the region of Peja in Kosovo, are summarized in Table 1 and Figure 2.

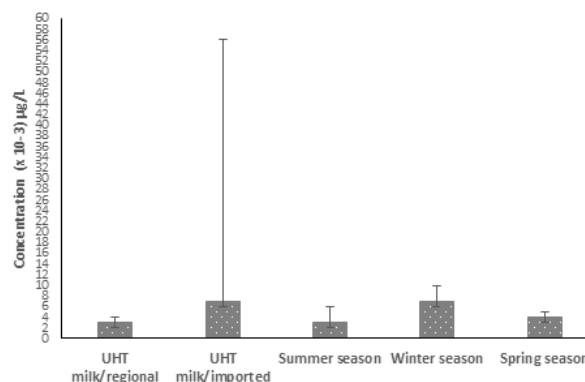


Figure 2. Comparison of mean AFM₁ levels in bulk tank milk samples of different seasons, and UHT regional and imported milk, in the region of Peja in Kosovo during 2021 - 2022

Out of a total of 160 samples examined, 75 (47%) samples were contaminated with AFM₁ at levels > LOD. Among them, 33% out of the total UHT milk samples were positive, and 52% of raw cow's milk samples.

The mean concentration of UHT regional milk samples was 0.003 µg/L and the corresponding mean value for UHT imported milk samples was 0.007 µg/L. The mean concentration for raw milk samples of summer 2021 was 0.003 µg/L, of winter 2021 was 0.007 µg/L, and the corresponding value for the spring samples of 2022 was 0.004 µg/L. The mean standard deviation of AFM₁ in UHT regional milk was 0.003 ± 0.001 µg/L, the corresponding value for UHT imported milk was 0.007 ± 0.049 µg/L; while the corresponding values for raw milk of summer, winter, and spring season are as follows, 0.003 ± 0.002 µg/L, 0.007 ± 0.003 µg/L, and 0.004 ± 0.001 µg/L, respectively. Only 1 (0.63%) sample exceeded the maximum tolerable limit, 0.05 µg/L, as set by the European Union Commission regulation number 1881/2006 [6], which belonged to UHT imported milk. In terms of seasonal distribution of positive samples of raw milk, the number of positive samples was slightly higher in the winter season. The highest level of contamination was detected in the

Table 1. Comparison of AFM₁ concentration in UHT milk and raw milk from the region of Peja in Kosovo

Type of samples/season	No. of samples/farms	Mean ± standard deviation, g/L	Median, µg/L	Maximum level of AFM ₁ , µg/L	No. (%) of samples exceeding LOD	No. (%) of samples exceeding the maximum level of 0.05 µg/L*
UHT milk (regional)	7	0.003 ± 0.001	0.004	0.008	3 (43%)	0
UHT milk (imported)	33	0.007 ± 0.049	0.013	0.165	10 (30%)	1 (3%)
Raw milk (summer season)	40	0.003 ± 0.002	0.007	0.015	17 (43%)	0
Raw milk (winter season)	40	0.007 ± 0.003	0.005	0.010	25 (63%)	0
Raw milk (spring season)	40	0.004 ± 0.001	0.002	0.006	20 (50%)	0
Total	160	0.004 ± 0.021	0.006	0.165	75 (47%)	1 (3%)

Legend: *European Commission (2006b). LOD Limit of detection (0.002 µg/L).

winter season samples, and in the UHT imported milk, but still this number was very low as shown in Table 1. However, mean and median were roughly within the same range for UHT and raw milk of different seasons. Based on some previous studies published in the last decade by some local authors, (Rama *et al.*, [16]; Camaj *et al.*, [2]; and Camaj *et al.*, [1]), Peja was the region with the highest number of positive samples as per the first author with 63.2%, and 40% as per the second author, respectively, which both reveal a relatively high overall contamination with AFM₁. This study shows that 52% of the samples were positive with AFM₁, but the mean value of the raw milk for this region is low (0.004 µg/L).

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4. Conclusions

- It can be concluded that the frequency of AFM₁ contamination of raw milk in the region of Peja in Kosovo in the time interval between summer 2021 and spring 2022, was relatively lower than the results reported on the previous studies for this region, and all the samples have been compliant with the European Union regulations for AFM₁.
- This shows that the farmers are more aware of the proper storage of animal feeding stuff, and the importance of regular monitoring of AFM₁ level in milk. As for the UHT imported milk samples, the results show that regular institutional monitoring is necessary.

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