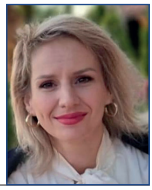
Occurrence of Aflatoxin M_1 in yogurt samples found in markets in Kosovo during spring 2023



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Abstract

Aflatoxin M_1 (AFM_1), a toxic byproduct of aflatoxin B_1 (AFB_1) produced by *Aspergillus* fungi, is a carcinogenic mycotoxin that can contaminate various agricultural commodities. It can be transferred from AFB_1-contaminated feed to milk and dairy products, including yogurt, posing a potential health risk to consumers. In spring 2023, a total of 74 yogurt samples were collected from the largest food suppliers in Kosovo for analysis, including samples produced in Kosovo and seven other countries: Albania, North Macedonia, Bosnia and Herzegovina, Slovenia, Greece, Italy, and Germany. A rapid and sensitive analytical method, Enzyme-linked immunosorbent assay (ELISA), was used for the analysis. The results of the study highlight discernible differences in the maximum tolerable levels of AFM_1 between the countries. More specifically, yogurt samples from Slovenia and Germany had lower levels than those from other countries. Additionally, the median levels of AFM_1 in samples from Slovenia and Germany were significantly lower. The mean concentrations of AFM_1 in yogurt samples from Kosovo and other countries were 0.071 μg/kg and 0.080 μg/kg, respectively. Out of all samples, 66 (89%) exceeded the maximum tolerable limit of 0.05 μg/kg. Among the exporting countries, Albania had the highest median AFM_1 level of 0.085 μg/kg and the highest maximum level of 0.195 μg/kg. Slovenia had the lowest median AFM_1 level, while Germany had the lowest maximum AFM_1 level. All samples from Albania, Greece, and Bosnia and Herzegovina exceeded the maximum tolerable limit. High prevalence was also observed in samples from Kosovo, North Macedonia, and Slovenia. Considering the average daily consumption of about 250 grams of yogurt, and the total median value of Aflatoxin M_1 concentration (0.071 μg/kg), the estimated daily intake was calculated to be 0.017 μg. These findings highlight the importance of monitoring and enforcing regulatory limits to ensure yogurt safety and to protect public health. Efforts should be focused on mitigating AFM_1 contamination and implementing measures to minimise its presence in dairy products, especially in regions where levels exceed the established limits.

Key words: *yogurt; ELISA; aflatoxin M_1; food; contamination*

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Abbreviations

AFM_1	Aflatoxin M_1
AFB_1	Aflatoxin B_1
ELISA	Enzyme-linked immunosorbent assay

Introduction

Aflatoxin M_1 (AFM_1) is a toxic metabolite of aflatoxin B_1 (AFB_1), a carcinogenic mycotoxin produced by Aspergillus fungi that can contaminate various agricultural commodities (IARC, 2002). AFM_1 can be transferred from AFB_1-contaminated feed to milk and dairy products, including yogurt, posing a potential health risk for humans who consume them (Prandini et al., 2009). Approximately 0.3–6.2% of AFB_1 is converted into metabolised AFM_1 and excreted in milk, depending on various factors (Iqbal et al., 2015). The International Agency for Research on Cancer (IARC) has classified AFM_1 with AFB_1 as a Group 1 carcinogen (IARC, 2002). Studies have shown that the presence of AFM_1 in milk and milk products, including yogurt, is a health issue due to their regular consumption by all age groups (Iqbal et al., 2015). The main harm of AFM_1 is its carcinogenicity and mutagenicity, which can destroy human and animal liver tissue, resulting in hepatocarcinoma and even death. AFM_1 alone can also cause damage to DNA by covalently binding to it, which may enhance the genotoxicity already caused by AFB_1 (Saha Turna et al., 2021). The presence of AFM_1 in milk and dairy products has been known for several decades and is an important problem worldwide, especially for developing countries (Iqbal et al., 2015).

Aflatoxin-producing fungi are found in areas with hot, humid climates and aflatoxins in food are a result of both pre- and post-harvest fungal contamination (EFSA et al., 2020). Climate change is anticipated to impact the presence of aflatoxins in food in Europe. Aflatoxin M_1 has stable physical and chemical properties and is not destroyed by pasteurisation. According to Iha et al. (2013), processing and storage have little effect on AFM_1 content in milk and milk products, with total AFM_1 mass in milk being reduced by 3.2% in cheese and 6% in yogurt (pH 4.4).

The European Commission has specified that AFM_1 in milk should not exceed 0.05 μg/kg (European Commission, 2006). Kosovo has adopted several EU regulations and standards for food safety and quality, including those related to dairy products such as yogurt. Dairy products are an important part of Kosovo's diet and economy, with significant annual per capita consumption of milk and cheese (Kosovo Agency of Statistics, 2018). In Kosovo, only a few studies have been conducted on AFM_1 in milk in recent years, and no research has been published on the presence of AFM_1 in yogurt. According to a 2016 study in Kosovo by Camaj et al. (2018), a high percentage of milk samples were found to be non-compliant with maximum AFM_1 levels. This suggests that efforts are needed to reduce contamination levels of aflatoxin B_1 in cow feed in Kosovo. In the Balkan region, several studies have been conducted over the past ten years on the incidence of aflatoxin M_1 in milk and dairy products (Camaj et al., 2018; Ilievska et al., 2022; Topi et al., 2022), indicating further efforts are needed to reduce the contamination levels of AFB_1 in cow feed, and regular monitoring of the milk is needed.

The objective of this research was to assess the occurrence of AFM_1 in yogurt samples found in markets and major food suppliers in Kosovo during spring 2023. These samples were produced in Kosovo and in countries exporting to Kosovo.

This study will contribute to safeguarding consumer health and promoting the production and consumption of safe and high-quality yogurt.

Materials and methods

Sample Collection

Out of this total, the 74 samples of yogurt were collected from markets (largest food suppliers) in Kosovo during spring 2023, for analysis of the quantity of AFM_1 toxin. Of the 74 samples, 40 samples were produced in Kosovo and 34 in other countries: North Macedonia (5), Albania (7), Bosnia and Hercegovina (6), Slovenia (6), Greece (6), Italy (2), and Germany (2). The samples were tested immediately after collection.

Laboratory analyses

The quantitative analysis of AFM_1 in the yogurt samples was done using enzyme-linked immunosorbent assay (ELISA) method, one of several methods used for the detection of AFM_1 in dairy products. It offers many advantages including a shorter analysis time, simultaneous analysis of many samples, limited use of organic solvents, absence of complicated sample preparation steps, simple analytical procedure compared to long-lasting and expensive chromatographic techniques (Jukić et al., 2020). To test AFM_1, we used the MEIZHENG Bio-Tech Co. Aflatoxin M_1 ELISA Test Kit, following the manufacturer`s instructions, and summarised as follows. Each testing reagent kit offered a certificate with the validation results (Table 1). While the specific ELISA protocol was not validated in our laboratory for this determination, it is important to note that this method is well-established and routinely used in our lab for aflatoxin M_1 testing, and the reagent kit offered a validation certificate. Our lab has extensive experience with this methodology, ensuring reliability and reproducibility of results.

All samples were tested at room temperature (20-25°C) immediately after collection. The dilution factor was 1. For testing, 1 g of each sample was measured and diluted with 4 mL of sample diluting buffer I, and vortexed for 2 minutes. Then samples were centrifuged for 5 minutes at 4000 rpm. For the assay, 50 µL of the sample dilution was measured.

The principle of the Aflatoxin M_1 ELISA Test Kit is an indirect competitive enzyme-labelled immunoassay. The aflatoxin M_1 antigen is precoated on the wells. The aflatoxin M_1 in the sample competes with the aflatoxin M_1 antibody with aflatoxin M_1 antigen on the well, while the AFM_1 antibody combines with the enzyme conjugate. Then the substrate solution is pipetted to the wells to convert the colour. The colour of the unknown samples is compared to the colour of the standards and the aflatoxin M_1 concentration is derived. The absorbance values were obtained using a plate reader set at 450 nm, and the level of AFM_1 was calculated using a logarithmic standard curve, and the average of duplicates was used as the result. According to the manufacturer's certificate, sensitivity was 0.015 µg/kg (the same as the LOD of this kit for raw milk determination), which seems to suggest it should be capable of detecting aflatoxin M_1 at levels lower than the LOD. Sensitivity is usually the concentration of the second standard (Table 1). Recovery was 100%±30%, and the precision for intra-lab assay was CV% <10%.

In the current study, it is essential to note that the Limit of Detection (LOD) of the employed kit for fermented milk and milk beverage is 0.075 µg/kg, which is higher than the maximum tolerable limit of aflatoxin M_1 in yogurt as set by the

regulatory guidelines (0.05 μg/kg). Interestingly, we observed results that were lower than the specified Limit of Detection (LOD) of the kit (0.075 μg/kg). These results indicate that some of the yogurt samples have very low or even undetectable levels of aflatoxin M_1.

Aflatoxin M_1 ELISA Test Kit standard summary

Table 1 provides the absorbance values A_{450}, B/B0 ratios, and CV% values, for different concentrations of Aflatoxin M_1 standards used in the ELISA test Kit validation, as provided on the certificate of analysis by the manufacturer. These values serve as reference points for comparing the results obtained from samples tested with the Aflatoxin M_1 ELISA Test Kit.

CV% (Coefficient of Variation) is calculated as CV= (SD/μ) x 100%, where SD is the standard deviation of the replicates (two replicates for each concentration of the standard), and μ is the average of replicates.

Statistical analysis

Statistical analysis was performed using SPSS. The results were grouped into two categories by country of production (Kosovo vs. other countries). The results obtained for the AFM_1 content were expressed as mean values with standard deviation (SD), as median, and as maximum concentration of AFM_1.

We used statistical tests to determine if there were any significant differences between the groups. For two groups,

Table 1. Aflatoxin M1 ELISA Test Kit validation results.

Aflatoxin M_1 (ng/kg) Negative control	A_{450}	B/B_0	CV%
0	1.836	100.0	2.1
15	1.410	76.2	4.2
45	0.901	49.1	3.7
150	0.356	19.4	0.4
500	0.090	4.9	3.1

A_{450} =Average absorbance using 450 nm primary filter and 630 nm differential filter

Table 2. AFM_1 concentration in yogurt produced in Kosovo and yogurt imported from other countries.

Origin of yogurt samples	*N*	Median μg/kg	Maximum level of AFM_1 μg/kg	No. (%) of samples exceeding LOD	No. (%) of samples exceeding the maximum level of 0.05 μg/kg*
Kosovo	40	0.070	0.110	16 (40%)	37 (92%)
Other countries	34	0.072	0.195	11 (32%)	29 (85%)
Total	74	0.071	0.195	27 (36%)	66 (89%)

*European Commission (2006b): LOD Limit of detection (0.075 μg/kg)

we used student's t-test and for three or more groups, we used One-Way Analysis of Variance (ANOVA) with a significance level of $P < 0.05$ for both tests. To confirm the outcomes of the independent t-test, we used effect size as an additional variable (Dankel et al., 2017). Effect size is a measure of the disparity between two group means (Lakens, 2013). Both substantive importance (effect size) and statistical importance (P value) must be presented to interpret the findings (Sullivan and Feinn, 2012).

Results and discussion

Table 2 presents the analysis of yogurt samples for the presence of AFM_1 (in µg/kg). The samples are categorised into two groups by country of production, i.e., Kosovo and imported from other countries.

Out of these samples, 40 samples of yogurt produced in Kosovo were tested and had a median AFM_1 level of 0.070 µg/kg, with a maximum level of 0.110 µg/kg. Of these, 16 samples (40%) exceeded the LOD (0.075 µg/kg) and 37 (92%) exceeded the maximum level of 0.05 µg/kg.

In comparison, 34 samples of yogurt produced in other countries were tested and had a median AFM_1 level of 0.072 µg/kg, with a maximum level of 0.195 µg/kg. Of these, 11 samples (32%) exceeded the LOD (0.075 µg/kg) and 29 (85%) exceeded the maximum level of 0.05 µg/kg.

In total, out of the 74 samples tested, the median AFM_1 level was 0.071 µg/kg and the maximum level was 0.195 µg/kg. Of all samples, 27 (36%) exceeded the LOD and 66 (89%) exceeded the maximum level of 0.05 µg/kg.

Table 3 shows the corresponding values of AFM_1 for the samples produced and imported from seven other countries.

Table 3 shows that yogurt produced in Albania had the highest median AFM_1 level of 0.085 µg/kg and the highest maxi-

Table 3. AFM_1 concentration in yogurt imported from other countries.

Origin of yogurt samples	*N*	Median µg/kg	Maximum level of AFM_1 µg/kg	No. (%) of samples exceeding LOD	No. (%) of samples exceeding the maximum level of 0.05 µg/kg*
Albania	7	0.085	0.195	5 (71%)	7 (100%)
North Macedonia	5	0.070	0.075	0	4 (80%)
Bosnia and Hercegovina	6	0.090	0.150	3 (50%)	6 (100%)
Greece	6	0.072	0.100	2 (33%)	6 (100%)
Slovenia	6	0.057	0.065	0	4 (67%)
Italy	2	0.077	0.110	1 (50%)	1 (50%)
Germany	2	0.042	0.055	0	1 (50%)
Total	34	0.070	0.195	11 (32%)	29 (85%)

*European Commission (2006b): LOD Limit of detection (0.075 µg/kg)

mum level of 0.195 μg/kg among all countries. All seven samples (100%) exceeded the maximum level of 0.05 μg/kg. In comparison, yogurt produced in North Macedonia had a lower median AFM_1 level of 0.070 μg/kg and a lower maximum level of 0.075 μg/kg. None of these samples exceeded the LOD and four (80%) exceeded the maximum level of 0.05 μg/kg. Yogurt produced in Bosnia and Herzegovina had a median AFM_1 level of 0.090 μg/kg, which is higher than that of North Macedonia but lower than that of Albania. The maximum level was 0.150 μg/kg, which was also higher than that of North Macedonia but lower than that of Albania. All six samples (100%) exceeded the maximum level of 0.05 μg/kg.

Yogurt produced in Greece had a median AFM_1 level of 0.072 μg/kg and a maximum level of 0.100 μg/kg, both lower than those of Albania and Bosnia and Herzegovina but higher than those of North Macedonia. All six samples (100%) exceeded the maximum level of 0.05 μg/kg. Yogurt produced in Slovenia had the lowest median AFM_1 level among all countries at 0.057 μg/kg and a low maximum level of 0.065 μg/kg. None of these samples exceeded the LOD and four (67%) exceeded the maximum level of 0.05 μg/kg. Yogurt produced in Italy had a median AFM_1 level of 0.077 μg/kg and a maximum level of 0.110 μg/kg, both higher than those of Slovenia but lower than those of Albania, Bosnia and Herzegovina, and Greece. Yogurt produced in Germany had the lowest maximum AFM_1 level among all countries at 0.055 μg/kg and a low median AFM_1 level of 0.042 μg/kg, which is lower than the median of all other countries.

In total, of the 34 samples tested from these countries, the median AFM_1 level was 0.070 μg/kg and the maximum level was 0.195 μg/kg.

According to the data calculated in Table 4, the findings of this study revealed that no statistically significant disparities ($\bar{x}_{Kosovo}$ = 0.071 μg/kg, $\bar{x}_{other\ countries}$ = 0.080 μg/kg, t=-0.1.307, *P*=0.198) were observed between Kosovo and other countries with respect to AFM_1 concentrations. The mean concentrations of Aflatoxin M_1 (AFM_1) in the analysed yogurt samples were 0.071 ± 0.018 μg/kg and 0.080± 0.037 μg/kg for yogurt from Kosovo and other countries, respectively.

The current study aimed also to investigate the variations in average levels of Aflatoxin M_1 (AFM_1) in yogurt across seven distinct countries that import yogurt into Kosovo (Table 5). The findings from this study highlight discernible differences in the maximum tolerable levels of AFM_1 among countries. More specifically, yogurt samples from Slovenia and Germany had lower levels than those from other countries. Additionally, the median levels of AFM_1 in samples from Slovenia and Germany were significantly lower. The mean AFM_1 levels were as

Table 4. The mean concentrations of Aflatoxin M_1 (AFM_1) of yogurt produced in Kosovo and imported from other countries.

	Kosovo	Other countries			
	Mean μg/kg	Mean μg/kg	Mean Difference	T-value	Significance
AFM_1 concentration	0.071	0.080	-0.009	-0.1.307	0.198

Table 5. The mean concentrations (μg/kg) of Aflatoxin M_1 (AFM_1) of yogurt samples imported from distinctive countries

	Albania	North Macedonia	Bosnia and Hercegovina	Greece	Slovenia	Italy	Greece
	x ± σ	x ± σ	x ± σ	x ± σ	x ± σ	x ± σ	x ± σ
AFM_1 concentration	0.11 ± 0.05a	0.08 ± 0.04c	0.08 ± 0.17b	0.07 ± 0.01e	0.05 ± 0.00f	0.07 ± 0.04d	0.04 ± 0.01g

follows: Albania (0.11 ± 0.05 μg/kg), North Macedonia (0.08 ± 0.04 μg/kg), Bosnia and Hercegovina (0.08 ± 0.17 μg/kg), Greece (0.07 ± 0.01 μg/kg), Slovenia (0.05 ± 0.00 μg/kg), Italy (0.07 ± 0.04 μg/kg), and Germany (0.04 ± 0.001 μg/kg).

Means that share the same letter are significant.

A two-year survey of aflatoxin M_1 in milk marketed in Albania, by Topi et al. (2022) found that AFM_1 was detected in 62 out of 119 milk samples (52.10%) and that AFM_1 contamination in pasteurised milk was higher than that in UHT milk samples (59.68% and 43.86%, respectively). Seven milk samples (5.88%) contained AFM_1 at levels exceeding the European Union maximum residue permitted level. A study published in 2022 (Ilievska et al.) revealed a high incidence of AFM_1 in UHT milk and dairy products marketed in North Macedonia. According to this study, the highest incidence of AFM_1 was found in yogurt samples (93.8%), with 178 yogurt samples tested.

Calculating Risk Assessment for aflatoxin M_1 in yogurt

To estimate the exposure to aflatoxin M_1 from yogurt, we need to calculate the estimated daily intake based on consumption patterns and the concentration of aflatoxin M_1 detected in yogurt. The formula to calculate the estimated daily intake is as follows:

Estimated Daily Intake (in micrograms) = average yogurt consumption (in grams) x aflatoxin M_1 concentration (in micrograms per kilogram) /per body weight

The Dietary Guidelines for Americans 2020–2025 recommends 3 cups of dairy per day for anyone over the age of 9 years. Each cup of yogurt counts as 1 cup of dairy, or about 250 grams daily. Based on this recommendation, if we consider that the average yogurt consumption per day is one cup, or about 250 grams, and if we take the total median value (0.071 μg/kg) as per aflatoxin M_1 concentration, then the estimated daily intake would be as follows:

Estimated Daily Intake (in micrograms) = 0.25 kg x 0.071 μg/kg = 0.017 μg

According to this assumption, if a grown person consumes one cup of yogurt per day, or about 250 grams, the estimated daily intake of aflatoxin M_1 from yogurt is 0.017 micrograms per body weight of the individual (in kg). Because no regulatory agency has set a tolerable daily intake (TDI) for AFM_1, it is not possible to compare exposure estimates to a TDI to determine at-risk populations (Saha Turna et al., 2021). According to Mollayusefian et al. (2021), the aflatoxin level in food commodities should be reduced to the lowest possible level.

Conclusions

In conclusion, the findings from this study highlight discernible differences in the maximum tolerable levels of AFM_1 among countries. More specifically, yogurt samples from Slovenia and Germany had lower levels than those from other countries. Additionally, the median levels of AFM_1 in samples from Slovenia and Germany were significantly lower.

The mean concentrations of AFM_1 in yogurt samples from Kosovo and other countries were 0.071 μg/kg and 0.080 μg/kg, respectively. Out of the total number of samples, 66 (89%) exceeded the maximal tolerable limit of 0.05 μg/kg. Among the countries that export to Kosovo, yogurt produced in Albania had the highest median AFM_1 level of 0.085 μg/kg and the highest maximum level of 0.195 μg/kg among all countries. Yogurt produced in Germany had the lowest maximum AFM_1 level among all countries at 0.055 μg/kg and the lowest median AFM_1 level of 0.042 μg/kg. All the samples from Albania, Greece, and Bosnia and Hercegovina exceeded the maximum tolerable limit for AFM_1. A high prevalence was shown in samples from Kosovo, North Macedonia, and Slovenia.

Considering the average yogurt consumption of one cup or about 250 grams per day and considering the total median value of aflatoxin M_1 concentration (0.071 μg/kg), the estimated daily intake was calculated to be 0.017 μg. The risk assessment for aflatoxin M_1 in yogurt was conducted to estimate the exposure to this toxin based on consumption patterns and its concentration in yogurt samples.

It is important to note that no regulatory agency has established a tolerable daily intake (TDI) for AFM_1. Therefore, comparing exposure estimates to a TDI to identify at-risk populations is not possible. However, existing research suggests that aflatoxin levels in food commodities should be minimised to the lowest possible level to ensure food safety.

These findings emphasise the importance of monitoring and enforcing regulatory limits to ensure the safety of yogurt and to protect public health. Efforts should be made to mitigate AFM_1 contamination and implement measures to minimise its presence in dairy products, particularly in regions where levels exceed the set limits. These measures will contribute to safeguarding consumer health and promoting the production and consumption of safe and high-quality yogurt.

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Pojavnost aflatoksina M_1 u uzorcima jogurta pronađenima na tržištu u Kosovu tijekom proljeća 2023.

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Aflatoksin M_1 (AFM_1), toksični nusproizvod aflatoksina B_1 (AFB_1) kojeg proizvode gljivice *Aspergillus* kancerogeni je mikotoksin koji može kontaminirati različite poljoprivredne proizvode; može se prenijeti iz hrane za životinje kontaminirane s AFB_1 na mlijeko i mliječne proizvode, uključujući i jogurt, predstavljajući potencijalni rizik za zdravlje potrošača. U proljeće 2023. godine prikupljena su ukupno 74 uzoraka jogurta od najvećih dobavljača hrane na Kosovu za analizu, uključujući uzorke proizvedene u Kosovu i u sedam drugih zemalja: Albaniji, Sjevernoj Makedoniji, Bosni i Hercegovini, Sloveniji, Grčkoj, Italiji i Njemačkoj. Za analizu je rabljena brza i osjetljiva analitička metoda, enzimski povezani imunosorbentni test (ELISA). Rezultati studije ukazuju da nalazi ove studije naglašavaju velike razlike u maksimalno dopuštenim razinama AFM_1 između različitih zemalja. Točnije, u uzorcima

jogurta iz Slovenije i Njemačke uočene su niže razine od onih iz drugih zemalja. Uz to, srednje razine AFM_1 u uzorcima iz Slovenije i Njemačke bile su značajno niže. Srednje koncentracije AFM_1 u uzorcima jogurta s Kosova i iz drugih zemalja bile su 0,071 μg/kg, odnosno 0,080 μg/kg. Od svih uzoraka, 66 (89 %) bilo je više od dopuštenog ograničenja od 0,05 μg/kg. Od zemalja uvoznica, Albanija je imala najveću srednju razinu AFM_1 od 0,085 μg/kg i najveću maksimalnu razinu od 0,195 μg/kg. Slovenija je imala najnižu srednju razinu AFM_1, a Njemačka najnižu maksimalnu razinu AFM_1. Svi uzorci iz Albanije, Grčke i Bosne i Hercegovine bili su veći od maksimalno dopuštene razine. Visoka prevalencija zamijećena je i u uzorcima s Kosova, iz Sjeverne Makedonije i Slovenije. Razmatrajući prosječnu dnevnu konzumaciju od jedne čašice od oko 250 g jogurta i ukupnu srednju vrijednost koncentracije aflatoksina M1 (0,071 μg/kg), izračunat je procijenjeni dnevni unos od 17,75 μg. Ovi nalazi naglašavaju važnost nadziranja i provođenja regulatornih ograničenja kako bi se osigurala sigurnost jogurta i zaštitilo zdravlje ljudi. Potrebno je uložiti napore za smanjenje kontaminacije AFM_1 i provedbu mjera za smanjenje njegove prisutnosti u mliječnim proizvodima, posebice u regijama u kojima razine prekoračuju utvrđena ograničenja.

Ključne riječi: *jogurt, ELISA, aflatoksin M_1, hrana, kontaminacija*