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
Abbreviations

AFM<sub>1</sub> Aflatoxin M<sub>1</sub>
AFB<sub>1</sub> Aflatoxin B<sub>1</sub>
ELISA Enzyme-linked immunosorbent assay

Introduction

Aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) is a toxic metabolite of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), a carcinogenic mycotoxin produced by Aspergillus fungi that can contaminate various agricultural commodities (IARC, 2002). AFM<sub>1</sub> can be transferred from AFB<sub>1</sub>-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<sub>1</sub> is converted into metabolised AFM<sub>1</sub> and excreted in milk, depending on various factors (Iqbal et al., 2015). The International Agency for Research on Cancer (IARC) has classified AFM<sub>1</sub> with AFB<sub>1</sub> as a Group 1 carcinogen (IARC, 2002). Studies have shown that the presence of AFM<sub>1</sub> 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<sub>1</sub> is its carcinogenicity and mutagenicity, which can destroy human and animal liver tissue, resulting in hepatocarcinoma and even death. AFM<sub>1</sub> alone can also cause damage to DNA by covalently binding to it, which may enhance the genotoxicity already caused by AFB<sub>1</sub> (Saha Turna et al., 2021). The presence of AFM<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> content in milk and milk products, with total AFM<sub>1</sub> mass in milk being reduced by 3.2% in cheese and 6% in yogurt (pH 4.4).

The European Commission has specified that AFM<sub>1</sub> 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<sub>1</sub> in milk in recent years, and no research has been published on the presence of AFM<sub>1</sub> 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<sub>1</sub> levels. This suggests that efforts are needed to reduce contamination levels of aflatoxin B<sub>1</sub> 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<sub>1</sub> in milk and dairy products (Camaj et al., 2018; Ilievksa et al., 2022; Topi et al., 2022), indicating further efforts are needed to reduce the contamination levels of AFB<sub>1</sub> in cow feed, and regular monitoring of the milk is needed.

The objective of this research was to assess the occurrence of AFM<sub>1</sub> 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 Biotech 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 M₁.

**Aflatoxin M₁ ELISA Test Kit standard summary**

Table 1 provides the absorbance values A₄₅₀/ B/B₀ ratios, and CV% values, for different concentrations of Aflatoxin M₁ 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 M₁ 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 AFM₁ content were expressed as mean values with standard deviation (SD), as median, and as maximum concentration of AFM₁.

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

<table>
<thead>
<tr>
<th>Aflatoxin M₁ (ng/kg)</th>
<th>A₄₅₀</th>
<th>B/B₀</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>1.836</td>
<td>100.0</td>
<td>2.1</td>
</tr>
<tr>
<td>15</td>
<td>1.410</td>
<td>76.2</td>
<td>4.2</td>
</tr>
<tr>
<td>45</td>
<td>0.901</td>
<td>49.1</td>
<td>3.7</td>
</tr>
<tr>
<td>150</td>
<td>0.356</td>
<td>19.4</td>
<td>0.4</td>
</tr>
<tr>
<td>500</td>
<td>0.090</td>
<td>4.9</td>
<td>3.1</td>
</tr>
</tbody>
</table>

A₄₅₀ =Average absorbance using 450 nm primary filter and 630 nm differential filter

**Table 2. AFM₁ concentration in yogurt produced in Kosovo and yogurt imported from other countries.**

<table>
<thead>
<tr>
<th>Origin of yogurt samples</th>
<th>N</th>
<th>Median µg/kg</th>
<th>Maximum level of AFM₁ µg/kg</th>
<th>No. (%) of samples exceeding LOD</th>
<th>No. (%) of samples exceeding the maximum level of 0.05 µg/kg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kosovo</td>
<td>40</td>
<td>0.070</td>
<td>0.110</td>
<td>16 [40%]</td>
<td>37 [92%]</td>
</tr>
<tr>
<td>Other countries</td>
<td>34</td>
<td>0.072</td>
<td>0.195</td>
<td>11 [32%]</td>
<td>29 [85%]</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>0.071</td>
<td>0.195</td>
<td>27 [36%]</td>
<td>66 [89%]</td>
</tr>
</tbody>
</table>

*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 $\mu$g/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 $\mu$g/kg, with a maximum level of 0.110 $\mu$g/kg. Of these, 16 samples (40%) exceeded the LOD (0.075 $\mu$g/kg) and 37 (92%) exceeded the maximum level of 0.05 $\mu$g/kg.

In comparison, 34 samples of yogurt produced in other countries were tested and had a median AFM$_1$ level of 0.072 $\mu$g/kg, with a maximum level of 0.195 $\mu$g/kg. Of these, 11 samples (32%) exceeded the LOD (0.075 $\mu$g/kg) and 29 (85%) exceeded the maximum level of 0.05 $\mu$g/kg.

In total, out of the 74 samples tested, the median AFM$_1$ level was 0.071 $\mu$g/kg and the maximum level was 0.195 $\mu$g/kg. Of all samples, 27 (36%) exceeded the LOD and 66 (89%) exceeded the maximum level of 0.05 $\mu$g/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 $\mu$g/kg and the highest

<table>
<thead>
<tr>
<th>Origin of yogurt samples</th>
<th>$N$</th>
<th>Median AFM$_1$ level $\mu$g/kg</th>
<th>Maximum AFM$_1$ level $\mu$g/kg</th>
<th>No. (%) of samples exceeding LOD</th>
<th>No. (%) of samples exceeding the maximum level of 0.05 $\mu$g/kg$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albania</td>
<td>7</td>
<td>0.085</td>
<td>0.195</td>
<td>5 (71%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>North Macedonia</td>
<td>5</td>
<td>0.070</td>
<td>0.075</td>
<td>0</td>
<td>4 (80%)</td>
</tr>
<tr>
<td>Bosnia and Herzegovina</td>
<td>6</td>
<td>0.090</td>
<td>0.150</td>
<td>3 (50%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Greece</td>
<td>6</td>
<td>0.072</td>
<td>0.100</td>
<td>2 (33%)</td>
<td>6 (100%)</td>
</tr>
<tr>
<td>Slovenia</td>
<td>6</td>
<td>0.057</td>
<td>0.065</td>
<td>0</td>
<td>4 (67%)</td>
</tr>
<tr>
<td>Italy</td>
<td>2</td>
<td>0.077</td>
<td>0.110</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
</tr>
<tr>
<td>Germany</td>
<td>2</td>
<td>0.042</td>
<td>0.055</td>
<td>0</td>
<td>1 (50%)</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>0.070</td>
<td>0.195</td>
<td>11 (32%)</td>
<td>29 (85%)</td>
</tr>
</tbody>
</table>

$^*$European Commission (2006b): LOD Limit of detection (0.075 $\mu$g/kg)
The mean concentrations of Aflatoxin M$_1$ (AFM$_1$) of yogurt produced in Kosovo and imported from other countries.

<table>
<thead>
<tr>
<th></th>
<th>Kosovo</th>
<th>Other countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean µg/kg</td>
<td>0.071</td>
<td>0.080</td>
</tr>
<tr>
<td>Mean Difference</td>
<td>-0.009</td>
<td>-</td>
</tr>
<tr>
<td>T-value</td>
<td>-0.1307</td>
<td>0.198</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. The mean concentrations of Aflatoxin M$_1$ (AFM$_1$) of yogurt produced in Kosovo and imported from other countries.
Estimated Daily Intake (in micrograms) = average yogurt consumption (in grams) x aflatoxin M₁ 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 M₁ 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 M₁ 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 AFM₁, 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 Herzegovina 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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References

Occurrence of Aflatoxin M1 in yogurt samples found in markets in Kosovo during spring 2023

Pojavnost aflatoksina M1 u uzorcima jogurta pronađenima na tržištu u Kosovu tijekom proljeća 2023.

Aflatoksin M1 (AFM1), toksični nusproizvod aflatoksina B1 (AFB1) kojeg proizvode gljivice Aspergillus kancerogeni je mikotoksin koji može kontaminirati različite poljoprivredne proizvode; može se prenijeti iz hrane za životinje kontaminirane s AFB1 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 AFM1 između različitih zemalja. Točnije, u uzorcima jogurta pronađenima na tržištu u Kosovu tijekom proljeća 2023, ukupno 74 uzoraka jogurta, ukupno 13, ukazuje na previsoke AFM1 nivoje, što stavlja na promatranje potencijalnu opasnost za zdravlje potrošača. S druge strane, rezultati ukazuju na potrebu rješavanja problema kontaminacije AFB1 i AFM1 u prodanih mliječnim proizvodima, kao i na potrebu sistematičnijeg nadzora i analiza tog vahovog mikotoksina u Kosovu.

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jogurta iz Slovenije i Njemačke uočene su niže razine od onih iz drugih zemalja. Uz to, srednje razine AFM₁ u uzorcima iz Slovenije i Njemačke bile su značajno niže. Srednje koncentracije AFM₁ u uzorcima jogurta s Kosova i iz drugih zemalja bile su 0,071 µg/kg, odnosno 0,080 µg/kg. Od svih uzorca, 66 (89 %) bilo je više od dopuštenog ograničenja od 0,05 µg/kg. Od zemalja uvoznica, Albanija je imala najveću srednju razinu AFM₁ od 0,085 µg/kg i najveću maksimalnu razinu od 0,195 µg/kg. Slovenija je imala najnižu srednju razinu AFM₁, a Njemačka najnižu maksimalnu razinu AFM₁. 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 napre u zaobilazivanje kontaminacije AFM₁ i provedbu mjera za smanjenje njegove prisutnosti u mliječnim proizvodima, posebice u regijama u kojima razine prekoračuju utvrđena ograničenja.

Ključne riječi: jogurt, ELISA, aflatoksin M₁, hranj, kontaminacija