Aflatoxin M₁ in processed milk: Occurrence and seasonal variation with an emphasis on risk assessment of human exposure in Serbia

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Abstract. The objectives of this study were to assess aflatoxin M₁ (AFM₁) contamination in processed milk and dairy products, and to estimate the mean daily exposure of the adult Serbian population to AFM₁ due to milk consumption. A total of 1734 samples, comprising heat treated cow’s milk (n=1233), infant formulae (n=349), milk powder (n=94) and dairy drink (n=58), were analyzed for AFM₁ presence using an Enzyme-Linked Immunosorbent Assay (ELISA) commercial kit. Samples were collected from different regions of Serbia during four seasons each year during 2015 and 2016. The incidences of AFM₁ contamination were 77.8% with a mean level of 0.027 ± 0.03 μg/L (range of <0.005-0.278 μg/L) in samples collected in 2015, and 98.4% with a mean level of 0.039 ± 0.02 μg/L (range of <0.005-0.28 μg/L) in samples collected in 2016. The highest AFM₁ levels were measured in October 2015 (0.278 μg/L) and September 2016 (0.279 μg/L). Based on EU regulation, 214 (17.3%) milk samples exceeded the maximum residue limit (0.05 μg/L). The estimated daily intake (EDI) of AFM₁ during different seasons of year for males and females was in the range of 0.022-0.330 (mean 0.20) ng/kg/bw/day and 0.022-0.30 (mean 0.18) ng/kg/bw/day, respectively. The calculated EDI indicate a public health concern due to the carcinogenic effects of AFM₁.

1. Introduction

Aflatoxin M₁ (AFM₁) is the most significant toxin in milk and dairy products. This compound is the hydroxylated form of the aflatoxin B₁ (AFB₁) that is formed in the liver of lactating animals, and is excreted into the raw milk by dairy cows when animals have been fed with feedstuffs containing AFB₁ [1]. Aflatoxins are potent, naturally occurring carcinogenic mycotoxins with a genotoxic effect and chronic and acute liver toxicity, especially in combination with hepatitis [2]. Since AFM₁ is stable at high temperatures (≥250 °C) and so cannot be removed from milk by heating processes, this toxin can be transferred into the milk products. Thus, AFM₁ contamination can be a substantial public health concern. AFM₁ has been classified as a Group 2 human carcinogen by the International Agency for Research on Cancer [3]. For this reason, and taking into account the significance of milk and milk products in the human diet (especially for children), the maximum allowed levels of AFM₁ are strictly regulated worldwide and range from 0 to 1.0 μg/kg. In Serbia, high incidences of detectable AFM₁ in milk have resulted in periodic changing of the maximum residue levels (MRL) for AFM₁ set by the Serbian Government [4].

Despite the importance that the presence of AFM₁ in milk has for public health concerns, in Serbia, the risks associated with AFM₁ in dairy products have yet to be fully understood. Assessment of risk...
of exposure to AFM1-contaminated milk and dairy products, is one of the most useful methods to evaluate the severity and probability of liver cancer risk in toxicological studies [5]. Therefore, the aims of the present survey were to determine the incidence of detectable levels of AFM1 in milk during different seasons, and to estimate the mean daily exposure of the adult Serbian population to AFM1 due to milk consumption.

2. Materials and Methods

2.1. Sample collection and preparation
A total of 1734 samples, comprising heat treated cow’s milk (n=1233), infant formulae (n=349), milk powder (n=94) and dairy drink (n=58) were obtained in the period from January 2015 to December 2016 from dairy producers during occasions of self-controls or official controls. Samples were stored at 2-8 °C or frozen at -20 °C until further analysis of AFM1. Milk samples were prepared according to the ELISA kit manufacturer’s instructions. Samples were centrifuged for 10 min at 3500 g at 10 °C. After centrifugation, the upper cream layer was completely removed by aspirating through a Pasteur pipette. Skimmed milk was used directly in the test (100 µl per well).

2.2. Enzyme-linked immunosorbent assay (ELISA) analysis
The ELISA test procedure was performed using the Aflatoxin M1 ELISA kit (Tecna S.r.l., Italy). Preparation of the samples and ELISA test procedure were performed according to the instruction provided by the manufacturer. The detection limit of the method was 0.005 µg/kg. In the case of AFM1 levels higher than 250 µg/kg, samples were diluted with sample dilution buffer and reanalyzed. Relative standard deviation of reproducibility was 6%. Recovery was 110%.

2.3. Exposure assessment
Estimated daily intake (EDI; ng/kg body weight (bw/day)) was calculated through a deterministic method [6]. For exposure calculations, levels of AFM1 below LOD were assumed to be equal to half the LOD for derived mean concentrations (0.0025 µg/kg) [7]. The daily intake level of milk and mean body weight for males and females was estimated based on data retrieved from Kos et al. [8]. Results were expressed as ng/kg bw/day.

3. Results and Discussion
The occurrence of AFM1 contamination in heat-treated cow milk and dairy products and EDI due to consuming AFM1 contaminated milk, collected during four seasons of 2015-2016, are presented in table 1 and figure 1.

3.1. Occurrence of AFM1
AFM1 was detected in 90.6% (117 out of 1233) of milk samples at concentrations ranging from 0.005 to 0.28 µg/kg, with a mean level of 0.035±0.029 µg/kg. Individual results by month (data not shown) showed the highest incidence of AFM1 in 2016 (98.4%) with a mean concentration 0.039±0.02 µg/kg, in comparison to 2015 (77.8%) with a mean concentration 0.027±0.03 µg/kg. However, the range of AFM1 concentrations between years was similar; 0.005-0.28 µg/kg. The highest concentration, which exceeded the MRL, was determined in winter and spring season (0.279 µg/kg). Statistical analysis of the data showed significant differences between periods of investigation in the level of AFM1 in heat-treated cow’s milk (p < 0.001) (table 1).
These differences are presumably due to contamination of feeds as influenced by local weather conditions during pre-harvest and harvest stage. Increased levels of AFM1 in Serbian milk since 2013 were most probably the consequence of feeding corn contaminated with AFB1. AFB1 contamination is prevalent in warm and humid climates and is reported in temperate countries following severe drought, particularly in cases of inadequate storage conditions [9]. Serbia has a continental to moderate continental climate with frequent, heavy rainfall. The temperatures (up to 40 °C) and relative
humidity (up to 80%) are high throughout the year. Under such climate conditions, a high incidence and levels of mycotoxins can be expected [10].

Table 1. The occurrence of AFM1 in different types of dairy products.

| Type of milk             | Year | N   | Incidence n (%) | Concentration of AFM1 (µg/kg) | Above MRL n (%) |
|-------------------------|------|-----|-----------------|-------------------------------|-----------------|
|                         |      |     |                 | Mean ± Sd                     | Range (min.-max.)| SRB n (%)[1,2] | EU n (%)[1] |
| Heat treated cow’s milk | 2015 | 468 | 364 (77.8)      | 0.027±0.03³                  | <0.005-0.278     | 12 (2.5)       | 43 (9.1)    |
|                         | 2016 | 765 | 753 (98.4)      | 0.039±0.02³                  | <0.005-0.28     | 2 (0.2)        | 171 (22.3)  |
| Sum of 2015 and 2016    | 1233 |    | 1117 (90.6)     | 0.035±0.02⁹                  | <0.005-0.28     | 14 (1.1)       | 214 (17.3)  |
| Infant formulae         | 2015 | 172 | 5 (2.9)         | 0.0086±0.0001                | <0.005-0.010    | 0              | 0           |
|                         | 2016 | 177 | 18 (10.2)       | 0.0012±0.0023                | <0.005-0.017    | 1 (0.5)        | 1 (0.5)     |
| Sum of 2015 and 2016    | 349  |    | 23 (6.6)        | 0.011±0.0025                 | <0.005-0.017    | 1 (0.2)        | 1 (0.2)     |
| Milk powder             | 2015 | 68  | 15 (22.1)       | 0.021±0.01                   | <0.005-0.035    | 0              | 0           |
|                         | 2016 | 26  | 10 (38.4)       | 0.013±0.008                  | <0.005-0.027    | 0              | 0           |
| Sum of 2015 and 2016    | 94   |    | 25 (26.6)       | 0.018±0.01                   | <0.005-0.035    | 0              | 0           |
| Dairy drink             | 2015 | 38  | 7 (18.4)        | 0.054±0.05                   | <0.005-0.147    | 0              | 3 (7.9)     |
|                         | 2016 | 20  | 6 (30.0)        | 0.010±0.0025                 | <0.005-0.013    | 0              | 0           |
| Sum of 2015 and 2016    | 58   |    | 13 (22.4)       | 0.034±0.04                   | <0.005-0.147    | 0              | 3 (5.2)     |

Means with same letters within the same column are significantly different at p < 0.001.
N – total number of analysed samples.
n – number of samples.
MRL 10.05; 2 0.25.

In our study, AFM1 was found in 23 out of 349 (6.6%) of infant formulae (levels ranged from <LOD to 0.017 µg/kg, mean 0.011±0.0025 µg/kg). The toxin incidence was higher in 2016 (18 out of 177, 10%) than those obtained in 2015 (5 out of 172, 3%). Regarding milk powder, the incidences of AFM1 contamination in samples collected from 2015 and 2016 were 22% (15 out of 68) and 38.4% (10 out of 26), with concentrations ranging from <LOD to 0.035 µg/kg and <LOD to 0.027 µg/kg, respectively. For the dairy drink samples, AFM1 was detected in 13 out of 58 (22.4%) samples, with the mean level of 0.034±0.04 µg/kg for these positive samples. The incidences of AFM1 contamination in samples collected in 2015 and 2016 were 7 out of 38 (18%) and 6 out of 30 (30%) with a range of <LOD to 0.147 µg/kg (mean 0.054±0.05 µg/kg) and <LOD to 0.013 µg/kg (mean 0.010±0.0025 µg/kg), respectively.

Based on EU Regulation No 466/2001, the maximum permitted level (MPL) for AFM1 in milk (raw milk, milk for the manufacture of milk based products and heat-treated milk) is 0.05 µg/L. According to this standard, 9.1% (43/468) in 2015 vs. 22.3% (171/765) in 2016, of milk samples had AFM1 levels that exceeded this limit. However, according to Serbian legislation, only 12 (2.5%) and 2 (0.2%) in 2015 and 2016 of milk samples exceeded the Serbian MRL, respectively. Moreover, in the present study, the AFM1 concentrations of three dairy drink samples was higher than 0.05 µg/kg. Since 2013, high levels of AFM1 in milk and dairy products have been detected in Serbia [8,11-16]. Overall, seasonal differences of AFM1 concentrations in milk and dairy products observed in this study also were recently reported in Serbia [1,16], and Croatia [17,18].

3.2 Exposure assessment - Estimated daily intake (EDI)
The EDI of AFM₁ for the adult Serbian population due to milk consumption during the period of the investigation is presented in figure 1. As shown in figure 1, the EDI in this study was higher during September 2015 to June 2016 than the amount recommended by Kuiper-Goodman (0.2 ng/kg bw/day) [19]. However, our study did not take the contribution of other dairy products into account.

![Figure 1](image)

**Figure 1.** EDI (ng/kg bw/day) for AFM₁ in heat-treated milk during seasons 2015-2016 (M-male, F-female).

Considering our results, it could be concluded that consumers are constantly exposed to this toxin through milk consumption. The average calculated EDI in our study was higher than the level reported by Torovic et al. [5], but lower than those reported by Skrbic et al. [11] or Kos et al. [8]. At the international level, on the basis of the mean concentrations of AFM₁ in milk and the milk consumption in the GEMS/Food regional diets [20], the EDI for the European diet was calculated as 0.11, for Latin American 0.058, for Far Eastern 0.20, for Middle Eastern 0.10, and for African diet as 0.002 ng/kg bw/day. Because of the carcinogenic potential of aflatoxins, JECFA [20] concluded that daily exposure, even to <1 ng/kg bw, contributes to the risk of liver cancer.

**4. Conclusion**

Considering the results obtained in this study, the EDI and potential hazard index, the unsuitably large intakes of AFM₁ by the Serbian population are higher than the recommended permissible intakes by international expert committees. Moreover, integrated prevention strategies at pre-harvest or post-harvest times, including further studies, in an attempt to identify and then control potential influencing factors, are necessary. Also, AFB₁ contamination of animal feeds and milk should be regularly monitored, particularly in regions where milk samples were previously contaminated above the legal limit.

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