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# MYCOTOXIN SURVEILLANCE IN DAIRY INDUSTRY: TRENDS AND IMPLICATIONS FROM A FIVE-YEAR STUDY IN NORTH MACEDONIA

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## ABSTRACT

The increasing demand for livestock production worldwide has heightened the importance of ensuring the safety of animal feed, with mycotoxins emerging as a significant hazard. These toxic secondary metabolites, produced by fungi on agricultural commodities, pose risks to both animal and human health, as well as to animal productivity and veterinary costs. Aflatoxin $B_1$ ($AFB_1$), a prominent mycotoxin, is anticipated to become a critical food safety issue, particularly in maize in Europe, where climate change may exacerbate its occurrence. This study aimed to summarize the prevalence of $AFB_1$ and its metabolite $AFM_1$ in dairy cow feed and milk in the Republic of North Macedonia from 2018 to 2022, evaluating the current situation and extrapolating $AFB_1$ levels in milk from $AFM_1$ findings. Samples were collected and analyzed using screening ELISA and confirmatory HPLC-FD methods. The results revealed persistent $AFM_1$ and $AFB_1$ contamination, with levels exceeding regulatory limits in some instances. Back-calculation was performed to estimate $AFB_1$ levels in the feed from $AFM_1$ values, highlighting discrepancies and the need for improved control measures. The study underscores the necessity for regular monitoring and control of aflatoxins in milk production stages and emphasizes the importance of proactive measures to mitigate the impact of mycotoxin contamination, particularly in light of climate change. These findings provide valuable insights into the ongoing mycotoxin contamination issue in dairy products, emphasizing the need for continuous surveillance and mitigation strategies to ensure food safety and protect public health.

**Key words:** *Mycotoxin contamination, Aflatoxin $B_1$($AFB_1$), Aflatoxin $M_1$($AFM_1$), North Macedonia, Milk production.*

## INTRODUCTION

The demand for livestock production is a key factor driving the demand for livestock feeds globally. Animal feed safety has become increasingly important, with mycotoxins being a significant hazard. Mycotoxins are toxic secondary metabolites produced by fungi that grow on agricultural commodities. Mycotoxins can have various impacts, including both animal and human health, reduced animal productivity, increased veterinary service costs, and feed disposal. Contamination of feeds can occur during both pre-harvest and post-harvest stages and can be carried over into food products, including milk (Xu et al., 2022; Leite et al., 2023). Aflatoxin, fumonisin, ochratoxin A, trichothecenes, and zearalenone are the most prevalent mycotoxins in animal feeds (Xu et al., 2022).

Aflatoxin $B_1$ ($AFB_1$) is predicted to become a critical food safety issue in maize in Europe, especially under the +2°C scenario, which is the most probable scenario of climate change expected in the coming years. This demands that we reinforce aflatoxin management efforts aimed at preventing human and animal exposure (Battilani et al., 2016). Acknowledging that it is metabolized into hydroxylated metabolite Aflatoxin $M_1$ ($AFM_1$), which is excreted in-cow milk, this issue presents a huge concern regarding the representation of milk and dairy products in the human diet (Antunović et al., 2022).

The European Commission has established maximum limits (ML) for the presence of these mycotoxins in raw cereals and derived products intended for human consumption: 2 µg/kg for $AFB_1$, 4 µg/kg for sum of aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$), ML of 50 ng/kg $AFM_1$ in milk (raw milk, heat-treated milk and milk for the manufacture of milk-based products) and 25 ng/kg for milk products for infants (EC, 2006). Due to unavoidable metabolization of $AFB_1$ into $AFM_1$, the ML of the toxin in feeds intended for milk-producing animals is limited to 5 µg/kg (EC, 2002).

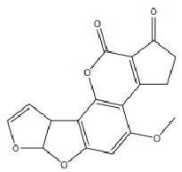
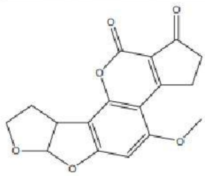
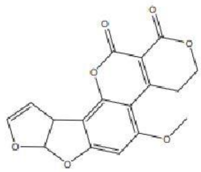
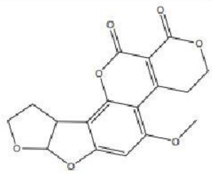
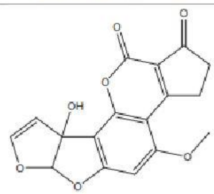
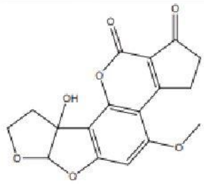
| Name | Aflatoxin B1 (AFB1) | Aflatoxin B2 (AFB2) | Aflatoxin G1 (AFG1) |
|---|---|---|---|
| Structure |  |  |  |
| CAS number | 1162-65-8 | 7220-81-7 | 1165-39-5 |
| Molecular formula | $C_{17}H_{12}O_6$ | $C_{17}H_{14}O_6$ | $C_{17}H_{12}O_7$ |
| Molecular weight | 312.3 g/mol | 314.3 g/mol | 328.3 g/mol |
| Name | Aflatoxin G2 (AFG2) | Aflatoxin M1 (AFM1) | Aflatoxin M2 (AFM2) |
| Structure |  |  |  |
| CAS number | 7241-98-7 | 6795-23-9 | 6885-57-0 |
| Molecular formula | $C_{17}H_{14}O_7$ | $C_{17}H_{12}O_7$ | $C_{17}H_{14}O_7$ |
| Molecular weight | 330.3 g/mol | 328.3 g/mol | 330.3 g/mol |

Figure 1. Chemical structure, CAS number, molecular formula, and molecular weight of aflatoxins
$B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $M_2$

The aim of the paper is to:

1. Summarize and present the prevalence of $AFB_1$ and $AFM_1$ mycotoxins in dairy cow feed and cow milk in the Republic of North Macedonia for the 2018-2022-time span.
2. Evaluate the current situation regarding the unveiled aflatoxins in milk and feed.
3. Extrapolate the expected $AFB_1$ levels in milk by performing back-calculation from $AFM_1$ findings.

## MATERIALS AND METHODS

*Sample collection*

Cow milk samples were collected from various locations in North Macedonia from 2018 to 2022, as part of the self-control survey programs conducted by dairy production facilities. A total of 3931 samples were collected for $AFM_1$ analysis and stored at +4°C for 24 hours or deep-frozen at temperatures below -20°C until analysis.

Dairy cow feed samples, totaling 147, were collected during the same time span (2018-2022) and kept at +4°C until analysis.

*Methods of analysis*

All milk samples were initially analyzed applying screening ELISA kit Immunoscreen (Tecna s.r.l, Trieste Italy) and Bio-Rad ELISA reader model 680 (Bio-Rad, Philadelphia, USA). Samples with values over 50 ng/kg were additionally analyzed with high-performance liquid chromatography with fluorescence detection (HPLC-FD), utilizing Waters Alliance 2695 system (Waters, Milford, MA, USA) (Dimitrieska-Stojković et al., 2014). The results from validation of screening and confirmatory methods for $AFM_1$ analysis were previously published (Dimitrieska-Stojković et al., 2014). Aflatoxin $B_1$ was analyzed utilizing HPLC-FD methodology on the aforementioned instrument, and the validation parameters were also previously published (Stojanovska-Dimzoska et al., 2022). Limit of detection (LOD) of the ELISA test for $AFM_1$ was 6.6 ng/kg, while the limit of quantification (LOQ) for $AFB_1$ was determined at level of 0.15 μg/kg.

*Calculation of extrapolated values of aflatoxin $B_1$ concentration in cattle feeds*

Based on the $AFM_1$ values obtained from raw milk samples, $AFB_1$ values in dairy cow feed were extrapolated through back calculation. This calculation was performed considering that, on average, approximately 1.3% of ingested $AFB_1$ is converted to $AFM_1$ (Dimitrieska-Stojković et al., 2016), following the formula proposed by Rastogi et al. (2004):

$$AFB_1\ (\mu g/kg) = [AFM_1\ (ng/kg) \times 100]/[1.3 \times 1000]$$

*Statistical analysis*

The obtained results for $AFM_1$ and $AFB_1$ underwent descriptive statistical analysis, including calculation of the percentage of positive samples, noncompliant samples, as well as the range and sample mean along with the associated standard deviation. Sample values below the limit of detection (LOD) for $AFM_1$ and below the limit of quantification (LOQ) for $AFB_1$ were set at zero for calculation purposes. The descriptive statistics were performed using Microsoft Excel 2016 MSO (version 16.0.4312.1000).

## RESULTS AND DISCUSSION

The results from the analysis of raw milk samples, along with the descriptive statistics for both screening and confirmatory analysis with HPLC-FD, are presented in Table 1. The positivity rate (samples over limit of detection, LOD) ranges from 22.4% to 42.5%, with a relatively constant non-compliance rate (samples over maximum limit, ML) below 3%, except for 2018 (3.9%). However, the highest confirmed $AFM_1$ level was unveiled in 2021, reaching 2300 ng/kg, representing approximately a 50-fold exceeding of the ML (EC, 2006). A probable reason for such a high $AFM_1$ level is the deficiency of official dairy feed control during the 2021 pandemic. In comparison to the results unveiled during the $AFM_1$ outbreak in 2013 (Dimitrieska-Stojković et al., 2016), the mean values determined for 2013 and 2021 are very similar, 14.3 and 14.2 μg/kg, respectively. Such findings for $AFM_1$ are further reflected in mycotoxin levels found in dairy products such as UHT milk, ice cream, yogurt, and cheese (Ilievska et al., 2022), imposing a moderate risk for the population from consumption of dairy products contaminated with $AFM_1$. Regarding the findings in the period 2014-2017 published by other authors (Santa et al., 2021) an increase in the non-compliance rate was observed in year 2018 when compared to year 2017. The results of our study provide evidence of the persistent $AFM_1$ issue in raw milk samples. However, consumers' risk is reduced because self-controls are performed in dairies before the production stage. A study published in Croatia covering the same time span, unveiled a non-compliance rate ranging from 0.48 to 4.32 % (Bilandžić et al., 2022). High positivity findings were also reported in a study from Hungary conducted in 2021 and 2022, totaling 9.4 % (Buzás et al., 2023).

Table 1. Distribution and descriptive statistics of $AFM_1$ concentration in raw cow milk in the period 2018 – 2022

| Sampling year | Number of samples | Positive samples[a] (%) | Min-Max (ng/kg) | Mean±SD[b] ng/kg | No. over ML[c] (%) |
|---|---|---|---|---|---|
| 2018 (total) | 1010 | 401 (39.7) | <6.6-184.0 | 12.4±19.4 | 39 (3.9) |
| 2019 (total) | 713 | 160 (22.4) | <6.6-322.6 | 8.1±21.5 | 21 (2.9) |
| 2020 (total) | 777 | 300 (38.4) | <6.6-215.6 | 9.7±17.7 | 20 (2.5) |
| 2021 (total) | 696 | 296 (42.5) | <6.6-2300 | 14.2±45.4 | 17 (2.4) |
| 2022 (total) | 735 | 242 (32.9) | <6.6-244 | 13.8±21.8 | 20 (2.7) |

[a]Over the LOD of ELISA method
[b]Total sample mean ± standard deviation
[c]Number of samples over the maximum limit (50 ng/kg)

Findings for $AFB_1$ for the period 2018-2022 are summarized in Table 2. The highest $AFB_1$ prevalence was identified in 2018 (70.6 %) with an average of 4.81 μg/kg and the highest determined $AFB_1$ level of 36.41 μg/kg. Nevertheless, in 2021 we unveiled the highest mycotoxin level for the whole study period, reaching 101.26 μg/kg. This year corresponds with the highest determined $AFM_1$ level of 2300 ng/kg (Table 1), although there is no proven traceability between these two results. Regarding the previous $AFB_1$ reports (Dimitrieska-Stojković et al., 2016), we observe similar non-compliance rates, between 2013 (13.4 %), and the years 2018 and 2022 (Table 2). In comparison to a study from other authors (Santa et al., 2021), our findings revealed higher positivity and non-compliance rates, as well as higher determined maximum values. The underlying reason for these non-compliant results could be explained by the heightened impact of climate change, leading to increased aflatoxin occurrence in food and feed (Kos et al., 2023). Over the past decade, mounting scientific evidence has increasingly urged caution regarding global warming and the emerging presence of mycotoxin-producing molds worldwide, even in regions with moderate climate conditions (Bilandžić et al., 2022).

Table 2. Distribution and descriptive statistics of $AFB_1$ concentration in dairy cow feed in the period 2018 – 2022

| Sampling year | Number of samples | Positive samples[a] (%) | Min-Max (µg/kg) | Mean±SD[b] µg/kg | No. over ML[c] (%) |
|---|---|---|---|---|---|
| 2018 (total) | 34 | 24 (70.6) | <0.15-36.41 | 4.81±8.24 | 5 (14.7) |
| 2019 (total) | 23 | 3 (13.0) | <0.15-4.38 | 0.36±1.01 | / |
| 2020 (total) | 22 | 5 (22.7) | <0.15-13.29 | 1.13±3.03 | 1 (4.5) |
| 2021 (total) | 33 | 8 (24.2) | <0.15-101.26 | 0.42±1.03 | 2 (6.1) |
| 2022 (total) | 35 | 12 (34.3) | <0.15-22.19 | 2.65±5.65 | 5 (14.3) |

[a]Over the LOQ of HPLC-FLD method
[b]Total sample mean ± standard deviation
[c]Number of samples over the maximum limit (5 µg/kg)

We back-calculated the anticipated levels of $AFB_1$ in dairy cow feeds from the occurrence data for $AFM_1$ (Table 3). The illustrated results in the table reveal a significant discrepancy between the extrapolated and found $AFB_1$ values. This discrepancy may arise from various reasons: notable difference in the number of tested results for $AFM_1$ in milk and $AFB_1$ in feed within the study; lack of traceability between the tested milk and feed samples; lower carry-over of $AFB_1$ from feed to milk compared to the 1.3% estimated in a previous study (Dimitrieska-Stojković et al., 2016); or a lower milk yield than the anticipated 40 liters per cow daily, resulting in a carry-over being lower than the EFSA estimation ranged 1-6% (EFSA, 2007). A previous study with proven traceability, estimated carry-over in the range of 0.22-3.74% (Dimitrieska-Stojković et al., 2016).
The disclosed results underscore the necessity for regular monitoring and control of aflatoxins in different stages of milk production: dairy cow feed, dairy farms, and the milk processing sector.

Table 3. Calculation of extrapolated values of aflatoxin $B_1$ concentration in cattle feed

| Year | Aflatoxin $M_1$ average (ng/kg) | Extrapolated Aflatoxin $B_1$ (µg/kg) | Found average Aflatoxin $B_1$ (µg/kg) |
|---|---|---|---|
| 2018 | 12.4 | 0.95 | 4.81 |
| 2019 | 8.1 | 0.62 | 0.36 |
| 2020 | 9.7 | 0.75 | 1.13 |
| 2021 | 14.2 | 1.09 | 0.42 |
| 2022 | 13.8 | 1.13 | 2.65 |

## CONCLUSION

In conclusion, our study highlights the persistent presence of $AFM_1$ and $AFB_1$ mycotoxins in dairy cow feed and milk samples in the Republic of North Macedonia from 2018 to 2022. The results underscore the importance of regular monitoring and control measures to mitigate consumer exposure to these toxins. The elevated levels observed, particularly in 2021, warrant urgent attention and intervention in feed control practices. Furthermore, our findings suggest a potential correlation between mycotoxin levels and climatic factors, emphasizing the need for proactive measures to address the impact of climate change on mycotoxin contamination. Overall, this study contributes valuable insights into the ongoing mycotoxin contamination issue in dairy products, emphasizing the importance of continuous surveillance and mitigation strategies to ensure food safety and protect public health.

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