Safety of milk and whey from Zlatibor region in relation to aflatoxin M₁ contamination: a seasonal study

D Milicevic¹, S Jankovic¹, K Muric², Z Petrovic¹, R Petronijevic¹, M Raseta¹ and J Djinovic-Stojanovic¹

¹ Institute of Meat Hygiene and Technology, Kacanskog 13, Belgrade, Serbia
² Zlatiborski eko agrar DOO, Cajetina, Serbia

E-mail: dragan.milicevic@inmes.rs

Abstract. The objectives of this study were to assess aflatoxin M₁ (AFM₁) contamination in cow and goat milk and whey samples collected from small dairy producers in rural areas of Zlatibor. The study involved a total of 60 samples of cow milk (n=15), cow whey (n=15), goat milk (n=15) and goat whey (n=15). In order to elucidate the distribution and stability of AFM₁ during milk processing, cheese manufacturing trials were performed using cow milk naturally contaminated with different concentrations of AFM₁ (<0.05 to >0.25 µg kg⁻¹). AFM₁ concentrations were measured using a validated immunoassay method (ELISA). None of the samples of milk or whey collected from areas of Zlatibor were contaminated with AFM₁. Contrary to that, in all of the analysed samples of whey obtained from contaminated cow milk, AFM₁ was detected. We conclude that whey contains 40-60% of the amount of AFM₁ present in milk. The results obtained indicate the intensification of dairy production and the supplementation with commercial feed such as maize could be risk factors that impact on the AFM₁ incidence in contaminated milk. In addition, our study demonstrates there is a potential health risk due to the consumption of whey obtained from contaminated milk.

1. Introduction
Milk and dairy products are very important in the human diet because of their positive influence on human health and ability to reduce the risks of cardiovascular disease, along with, particularly, the activity of conjugated linoleic acid (CLA) in inhibition of cancer, atherosclerosis and improvement of immune functions [1]. The dairy industry in Serbia is of great economic importance. According to official data, total milk production in Serbia is around 1506 million L/year (3505 L/per milked cow), with 97% of production being cow milk [2]. Thus far, cow milk is the most important type in Serbia, making up 96% of milk in the human diet, while goat and sheep milk production takes place on a small scale, and most of the milk is used by households or families.

Goat milk and its products are superior to cow milk, because goat milk is an excellent source of nutrients and is rich in high biological value proteins, essential fatty acids, high mineral bioavailability, volatile compounds (flavours, terpenes) and vitamin content [3]. Another aspect of the beneficial health effects of goat milk over other dairy species milks derives from the lower allergenicity of their proteins, greater digestibility and more bioactive components, meaning it is considered as a functional food [4]. Effort to encourage production and scientific research in this field will be positive for the goat dairy industry and for the commercialization of its products. Whey is a clear solution, a by-product of cheese
production. Whey is an excellent source of nutrients including lactose, protein, and minerals, but is often discarded or given to animals as a nutritional supplement. However, it is used in specialized nutrition for athletes, bodybuilders, elderly people, and people with obesity.

The hottest and driest period since 2010 in most of Serbia coincided with the most important growing phases of spring crops, causing substantial damage and losses in agricultural crop production manifested by high concentration of aflatoxins (AFs) in maize and, consequently, in feed and milk [5, 6]. AFM$_1$ is the most significant toxin in the dairy industry because its water-soluble compounds tend to bind to the protein fraction of milk, it is very heat-stable, and thus, it can contaminate dairy products, which usually contain a higher concentration of AFM$_1$ than the milk from which it was made [7].

AFs are one of the major etiological factors in the development of hepatocellular carcinoma (HCC). Although, the toxic effect of AFM$_1$ is approximately 10-fold less than AFB$_1$, AFM$_1$ has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer [8]. For this reason, and taking into account the significance of milk and milk products in the human diet (especially for children), the maximum permissible levels (MPL) of AFM$_1$ are strictly regulated worldwide. Serbia has set a MPL of 0.25 $\mu$g kg$^{-1}$ [9] which is between the MPL of the European Union (0.05 $\mu$g kg$^{-1}$) [10] and the internationally recognized limit (0.5 $\mu$g kg$^{-1}$) [11]. At the moment, this limit has been established following the ALARA (as Low as Reasonably Achievable) principle. Despite the fact that AFM$_1$ could also be 2-4 times higher in cheese than the levels initially present in milk [12], no specific AFM$_1$ limits have been set by the Serbian government for dairy products.

Serbia has also recorded a high estimated annual global burden of HCC cases attributable to AFM$_1$ exposure (0.01730 ± 0.019 for males versus 0.0166 ± 0.018 for females) [13]. There is a scarcity of systematic studies assessing the risk of exposure to AFM$_1$-contaminated milk and dairy products such as whey, and that is coupled with the fact that AFM$_1$ contamination of milk has been a serious problem in Serbia in recent years due to the climatic conditions. Therefore, the aims of the present survey were to: 1) investigate the occurrence of AFM$_1$ in raw cow, goat, and whey milk samples collected from small dairy producers from a mountain (Zlatibor) region, and; 2) assess the safety aspects, particularly due to whey consumption. This survey could be a useful scientific reference for the design of risk-based food safety surveillance and management for regional dairy production.

2. Materials and methods

2.1. Sample collection and preparation

The research was conducted during the autumn-winter season of 2018/2019 in Zlatibor region (South-Western Serbia 43° 45’ 0.048” N and 19° 42’ 56.023” E, 1,000 meters above sea level). During the study period, a total of 60 cow and goat milk and whey samples were collected from small dairy producers in rural areas of Zlatibor. In the autumn-winter season, all farms were feeding dairy stock mainly with haylage. Representative milk samples collected in plastic containers were transported under cold conditions to the laboratory of the Institute of Meat Hygiene and Technology and stored at-18°C until analysis. In order to elucidate distribution and stability of AFM$_1$ during milk processing, cheese manufacturing trials were performed from cow milk naturally contaminated with different concentrations of AFM$_1$ (<0.05 to >0.25 $\mu$g kg$^{-1}$). The AFM$_1$ content in raw cow milk and whey were determined as previously described [13]. 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 instructions provided by the manufacturer. The detection limit of the method (LOD) was 0.005 $\mu$g kg$^{-1}$. In the case of AFM$_1$ levels higher than 250 $\mu$g kg$^{-1}$, samples were diluted with sample dilution buffer and reanalyzed. Relative standard deviation of reproducibility was 6%. Recovery was 110%.
3. Results and discussion

The occurrence of AFM$_1$ contamination in raw cow and goat milk samples and in whey collected from small dairy producers in rural areas of Zlatibor are presented in Table 1. Concentrations of AFM$_1$ found in each whey sample and naturally contaminated milk from which whey was made are presented in Table 2 and Figure 1.

3.1. Occurrence of AFM$_1$

Based on our results, concentrations of AFM$_1$ in all cow and goat milk and whey samples were below the LOD. Therefore, the contamination level of AFM$_1$ is not considered a public health concern under Serbian or EU legislation. These results show a significantly different situation compared to the previous study from Serbia [13,14]. Increased levels of AFM$_1$ in Serbian milk since 2013 were most probably the consequence of feeding corn contaminated with AFB$_1$ [15]. It is considered that pasture-based dairy production systems (as occurred in our current study) present low risk [16], because AFs are not produced in pasture, are rarely found in forages and are usually not present in high enough concentrations in corn silage to be of concern. Indeed, the risk of AFs occurring in milk is believed to be largely confined to the feeding of grain-based concentrates. However, the carry-over rates of AFB$_1$ in milk depend on the animal species, but also can vary greatly depending upon nutritional, environmental, and physiological factors such as stage of lactation, systemic diseases and local (mammary) infections, level of AFB$_1$ in feed, rate of feed ingestion, and geographical and seasonal conditions [17].

| Type of milk | N  | Incidence n (%) |
|-------------|----|----------------|
| Cow Milk    | 15 | 0/15 (0)       |
| Cow Whey    | 15 | 0/15 (0)       |
| Goat Milk   | 15 | 0/15 (0)       |
| Goat Whey   | 15 | 0/15 (0)       |
| Total       | 60 | 0/60 (0)       |

N – total number of analysed samples; n – number of positive samples

3.2 Distribution and stability of AFM$_1$ during processing

Our study found there was a significant linear relationship between the amount of AFM$_1$ in contaminated milk and the AFM$_1$ concentration in whey (Fig. 1). During cheese manufacturing trials, 1L of cow milk produced 0.6 L of whey. The regression analyses between AFM$_1$ concentration in milk and whey show the AFM$_1$ concentrations in whey strongly depended on the AFM$_1$ concentrations in milk, and this is a good predictor of the AFM$_1$ concentration in whey. Most studies have reported that thermal treatments such as pasteurization and sterilization cannot change the level of AFM$_1$ in dairy products [18]. Based on the results of Škribić et al. [19], the level of AFM$_1$ in cheese depends on several factors including type of cheese, cheese-making procedures, ripening conditions (e.g., temperature, humidity and pH) and contamination level of milk. Moreover, the distribution of AFM$_1$ between curd and whey can be variable.

Our findings are in agreement with results of previous studies which concluded that during cheese production, 60% of the initial content of AFM$_1$ accumulates in the whey, while 40% of the AFM$_1$ remains in the curd or fresh cheese [12,17]. Accordingly, whey and cheese could be considered as one of the most important sources of AFM$_1$ among milk-based products. Beside milk, the presence of AFM$_1$ in other dairy products and the high intake of these products by the human population may have negative health implications for consumers. Based on the regulations of the EC and Codex Alimentarius...
Commission, the allowed MPL for AFM<sub>1</sub> in dairy products is 0.250 µg kg<sup>-1</sup> [20,21]. Except for the level regulated in milk, there is no MPL for AFM<sub>1</sub> contents in dairy products in Serbia.

**Table 2.** Concentrations of AFM<sub>1</sub> in whey and naturally contaminated cow milk from which whey was obtained

| Trial* | Milk µg kg<sup>-1</sup> | Total AFM<sub>1</sub> (µg) | Whey µg kg<sup>-1</sup> | Total AFM<sub>1</sub> (µg) |
|--------|-------------------------|---------------------------|-------------------------|---------------------------|
| 1      | 0.04                    | 0.11                      | 0.04                    | 0.06                      |
| 2      | 0.05                    | 0.12                      | 0.05                    | 0.08                      |
| 3      | 0.08                    | 0.20                      | 0.08                    | 0.12                      |
| 4      | 0.11                    | 0.27                      | 0.07                    | 0.10                      |
| 5      | 0.15                    | 0.37                      | 0.07                    | 0.10                      |
| 6      | 0.15                    | 0.38                      | 0.18                    | 0.27                      |
| 7      | 0.23                    | 0.59                      | 0.24                    | 0.37                      |
| 8      | 0.26                    | 0.65                      | 0.27                    | 0.40                      |
| 9      | 0.26                    | 0.66                      | 0.26                    | 0.39                      |
| 10     | 0.29                    | 0.73                      | 0.28                    | 0.42                      |

*Note: 2.5 litres of milk naturally contaminated with AFM<sub>1</sub> was used.

**Figure 1.** Relationship of AFM<sub>1</sub> concentration in whey and that in milk from which the whey was obtained

4. **Conclusion**

In the whole milk chain, AFM<sub>1</sub> carry over from feed to milk and transfer from milk to dairy products can be a potential threat both for farmers and dairy producers, who need to avoid, in milk, AFM<sub>1</sub> contamination that exceeds the legally permitted limit and to estimate the expected AFM<sub>1</sub> concentration in dairy products made from contaminated milk. The results of our cheese manufacturing trials confirm the AFM<sub>1</sub> concentration in whey is strongly dependent on the AFM<sub>1</sub> concentration in milk and could be
40-60% of the level initially present in milk. Moreover, the AFM$_1$ concentration in milk can be a good predictor of AFM$_1$ presence in the final products. Despite these encouraging results of investigation of milk and whey from the pastoral Zlatibor region, the presence of AFM$_1$, especially in cow milk, should never be underestimated or neglected, due to the unpredictability of climatic conditions.

Acknowledgment
This study was supported by project No. TR 31008 funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, and Innovation Fund Project, Grant No. 231.

References
[1] Trbovic D, Spiric D, Lukic M, Petrovic Z, Parunovic N, Djordjevic V and Milicevic D 2017 Fatty acid composition of cow’s milk: opportunities and challenges for Serbian dairy producers Meat Technol. 58(2) 118–24
[2] Statistical Yearbook of the Republic of Serbia 2018 Published and printed by: Statistical Office of the Republic of Serbia Belgrade. http://www.stat.gov.rs
[3] Clark S and García M B 2017 A 100-year review: Advances in goat milk research J. Dairy Sci. 100 10026–44
[4] Raynal-Ljutovac K, Lagriffoul G, Paccard P, Guillet I and Chilliard Y 2008 Composition of goat and sheep milk products: An update Small Ruminant Res. 79(1) 57–72
[5] Milicevic D, Nastasijevic I and Petrovic Z 2016 Mycotoxin in the food supply chain – implications for public health program J. Environ. Sci. Health. C. 34(4) 293–319
[6] Milicevic D, Lakicevic B, Petronjевич R, Petrovic Z, Jovanovic J, Stefanovic S and Jankovic S (2019a) Climate change: impact on mycotoxins incidence and food safety Theory and Practice of Meat Processing 4(1) 9–16
[7] Govaris A, Roussi V, Koidis P A and Botsoglou N A 2001 Distribution and stability of aflatoxin M1 during processing ripening and storage of Telemes cheese Food Addit. Contam. 18(5) 4
[8] IARC 2002 IARC monographs on the evaluation of carcinogenic risks to humans. In Traditional Herbal Medicines, some Mycotoxins, Naphthalene and Styrene (Vol 82) Lyon: IARC Press
[9] Serbian Regulation 2018 Maximum allowed contents of contaminants in food and feed Official Bulletin of the Republic of Serbia 22 401–6
[10] European Commission 2010 Commission regulation (EC) No 165/2010 of 26 February 2010 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins O. J. L50 8–11
[11] Codex Alimentarius 1995 Codex general standard for contaminants and toxins in food and feed Codex Standard 193-1995, Adopted 1995, Revised 1997, 2006, 2008, 2009. Amended 2010, 2012. Codex Secretariat; Rome, Italy
[12] Oruc H H, Cibik R , Yilmaz E and Kalkanli O 2006 Distribution and stability of Aflatoxin M1 during processing and ripening of traditional white pickled cheese Food Addit. Contam. 23(2) 190–5
[13] Miličević D, Špirić D, Radičević T, Velebit B, Stefanović S, Milojević L and Janković S 2017a A review of the current situation of aflatoxin M1 in cow’s milk in Serbia: risk assessment and regulatory aspects Food Addit. Contam. Part A DOI: 10.1080/19440049.2017.1363414
[14] Milicevic D, Spiric D, Jankovic S, Velebit B, Radicevic T, Petrovic Z and Stefanovic S 2017b Aflatoxin M1 in processed milk: Occurrence and seasonal variation with an emphasis on risk of human exposure in Serbia IOP Conf Ser Earth Environ Sci 85
[15] Milicevic D, Petronjевич R, Petrovic Z, Djinovic Stojanovic J, Jovanovic J, Balić T and Jankovic S 2019b Impact of climate change on aflatoxin M1 contamination of raw milk with a special focus on climate conditions in Serbia J. Sci. Food Agric. Article in press DOI:101002/jsfa9768
[16] Fink-Gremmels J 2008 Mycotoxins in cattle feeds and carry-over to dairy milk: a review Food Addit. Contam. Part A Chem. Anal. Control. Expo. Risk Assess. 25(2) 172–80
[17] Walte H G Schwake-Anduschus C Geisen R and Fritsche J 2016 Aflatoxin: food chain transfer from feed to milk J. Consum. Protect Food Safety 11 295-297 DOI 10.1007/s00003-016-1059-8

[18] Shahbazi Y 2017 Aflatoxin M1 Contamination in Milk and Dairy Products: Implications on Human Health. Chapter 19 In Nutrients in Dairy and Their Implications for Health and Disease http://dx.doi.org/10.1016/B978-0-12-809762-5.00019-X

[19] Škrbić B, Antić I and Živančev J 2015 Presence of aflatoxin M1 in white and hard cheese samples from Serbia Food Control 50 111–7

[20] Codex Alimentarius Commission 2001 Comments Submitted on the Draft Maximum Level for Aflatoxin M1 in Milk. The Netherlands: Hague: Codex Committee on Food Additives and Contaminants (33rd session)

[21] European Commission 2004 Commission regulation (EC) No 683/2004 O J L106 3-5