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AFLATOXIN M1 TRANSFER RATE FROM MILK INTO CHEESE AND WHEY DURING THE PRODUCTION OF HARD CHEESE*

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Summary: *The aim of this study was to investigate aflatoxin M1 (AFM1) transfer from naturally contaminated raw milk into cheese and whey, during the production of Livanjski cheese (hard cheese type). Raw milk samples were collected from 4 farms in Serbia. The samples were then tested for AFM1 content and were later used for hard cheese production. Four cheese samples were produced, and the matching whey samples were also collected. The milk samples included two samples containing AFM1 above the EU maximum level (0.077±0.009 and 0.118±0.008 µg/kg) and the other two samples below the maximum level of 0.05 µg/kg (0.021±0.002 and 0.034±0.004 µg/kg). Regarding AFM1 transfer into cheese from the milk samples containing AFM1 above the EU maximum level, the rate was approximately 4-fold (383% and 410%). On the other hand, in the cheese samples made from milk containing AFM1 below the EU maximum level, almost 10-fold levels of AFM1 content in milk were found (transfer rates of 934% and 961%). As for the whey samples, AFM1 levels were below the levels found in the milk samples (transfer rates of 78%, 74%, 68% and 57%). The difference in the transfer rates for the cheese made from the milk samples contaminated at different levels may indicate the possibility that the AFM1 transfer rate from milk into cheese depends on the content of this toxin in milk.*

Key words: *aflatoxin M1, transfer, milk, hard cheese, whey, HPLC-FLD.*

INTRODUCTION

The public in Serbia has been aware of the existence of aflatoxins (AFs) in milk and maize for the past few years. This was caused primarily by the socio-economic consequences of the severe milk contamination with aflatoxin M1 (AFM1) that began in 2013. The possible reasons might be that security blankets in crops at the pre-harvest and post-harvest level are not as strict as in developed countries, or that the developing countries have not accepted and assumed amenities as quickly as the developed countries (Williams et al., 2004; Lizárraga-Paulín et al., 2011). Indisputably, this is not entirely accurate for Serbia since it is an EU candidate and therefore is harmonizing the legislation with the EU. Nevertheless, Serbia still belongs to a group of developing countries according to the World Bank (2018).

International Agency for Research on Cancer classified aflatoxin B1 (AFB1) as carcinogenic (IARC, 2002). It has been known that if AFB1 is present in the diet of lactating animals, AFM1 would occur in milk (Cupid et al., 2004; Battacone et al., 2005; Lizárraga-Paulín et al., 2011). The conversion rate of AFB1 into AFM1 was found to be 1–3% (Herzallah, 2009). In milk of dairy cows AFM1 was found within 12–24 hours of AFB1 contaminated feed ingestion, while the highest levels were reached after a few days (Ayar, 2007). On the other hand, when contaminated feed is excluded from the diets, the AFM1 levels in the milk decreased to an undetectable level after 72 hours (Van Egmond, 1989; Gimeno, 2004; Özdemir, 2007). However, AF carry-over from feed into milk exponentially increases (Britzi et al., 2013). The aflatoxin carry-over ranges from 0.6 to 6% (Masoero et al., 2007)

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and can be up to 11.4% in case of high yielding cows (Britzi et al., 2013). Britzi et al. (2013) also suggested that in the case of high yielding cows with the average milk production of 45 kg and daily intake of 25 kg of dry matter, AFB1 needed to be below 1.4 μg/kg in cows' diet to ensure milk production with AFM1 levels lower than the EU legal limit.

When cheese is produced from AFM1-contaminated milk, this toxin will probably be present in the cheese made from this milk. This could be explained by the fact that AFM1 is bound to casein (Battacone et al., 2005), as well as whey proteins (Mendonça and Venâncio, 2005). Therefore, it is evident that AFM1 is likely to be present in the final products such as cheese, and its concentration therein would be several times higher than in the milk used for cheese production (Deveci, 2007; Manetta et al., 2009). Accordingly, AFM1 may also be present in other dairy products such as whey and products obtained from whey. AFM1 transfer rate in whey was found to be 40–60% (Govaris et al., 2001; Oruc et al., 2006; Deveci, 2007; Kamkar et al., 2008; Manetta et al., 2009). However, this rate can be higher, between 70% and 74% (Battacone et al., 2005), or much lower, between 17% and 27% (Lopez et al., 2001).

Given the impact AFM1 has on the consumers health, Codex Alimentarius Commission recommended the maximum level of 0.5 μg/kg (Codex Alimentarius Commission, 2001), although the limit of 0.05 μg/kg for raw milk, heat-treated milk and milk for the manufacture of milk-based products is set by the regulations in the European Union (European Commission, 2006). As for the hard cheese, only few countries in Europe (the Netherlands, Switzerland, Austria, Romania, Turkey and Italy) have set the maximum levels of AFM1, ranging from 0.2 μg/kg (the Netherlands) to 0.45 μg/kg (Argentina) (Anfossi et al., 2011). In Serbia, there are no regulations to control the presence of AFM1 in dairy products.

Maximum level (ML) in hard cheese has been set in some countries and it is usually 250 ng/kg, which rests on the premise that cheese is made from milk which is within the ML, and that the AFM1 levels may increase up to 5-times due to the moisture loss. Nevertheless, some countries have opted for a strategy of zero tolerance (Romania and Egypt) to ensure maximum protection of consumers (Anfossi et al., 2011). On the other hand, Italy raised the limit of AFM1 in hard cheese to 450 ng/kg to protect the production of Parmesan cheese, as a result of a high contamination of animal feed with AFB1 in 2004 (Anfossi et al., 2011).

A similar situation was also in Serbia regarding the regulation of AFM1 in raw milk. In 2011, the maximum level set in Serbia was 0.05 μg/kg (Serbian Regulation, 2011), but since then it has been changed several times. At this moment, the ML in Serbia is set to 0.25 μg/kg (Serbian Regulation, 2018).

Given the higher maximum permitted quantity of AFM1 in milk in Serbia in relation to the EU, and insufficient data on the transfer of AFM1 from milk into traditional dairy products, the aim of this study was to investigate AFM1 transfer from naturally contaminated raw milk into cheese and whey, during the production of a hard cheese type Livanjski cheese.

MATERIAL AND METHODS

Milk samples. Raw milk was collected during the spring of 2018 from 4 farms in Serbia that have less than 20 cows. The farms were selected by the AFM1 levels in milk, as a result of monitoring 23 farms. Approximately 20 liters of raw milk was taken, and the samples were labeled as sample numbers 1–4. The samples were collected directly from cooling tanks after morning milking. These samples were then tested for AFM1 content and were later used for hard cheese production.

Cheese production. Hard cheese type Livanjski cheese was produced from each milk sample resulting in 4 cheese and 4 whey samples. Cheese production was done according to a previously established technological procedure (Popović-Vranješ et al., 2013). Key steps in the production of hard cheese are presented in Table 1. AFM1 analysis of whey was performed within 36 hours of production, while cheese samples were analyzed after 60 days of maturation.

Aflatoxin M1 determination. Sample preparation was done using AflaStarTM M1 R-Immunoaffinity Columns (IAC) (Romer Labs Inc., Union, MO, USA), according to the manufacturer's procedure. Fifty ml of centrifuged milk/whey or diluted cheese extract was filtered through a quantitative filter paper (Filtros Anoia, Barcelona, Spain) and applied to the IAC. Flow rate of milk was approximately 1-3 ml/min. After the milk completely passed, IAC was rinsed with 20 ml of deionized water. The AFM1 was eluted with 4 ml of acetonitrile (Sigma Aldrich, Buchs, Switzerland). The eluate was collected and evaporated to dryness at 50 °C using gentle stream of nitrogen. AFM1 was derivatized by adding 200 μl of trifluoroacetic acid (Thermo Fisher Scientific, Cheshire, United Kingdom) and the same volume of n-hexane (Merck, Darmstadt, Germany) to the residue and to the AFM1 standards, vortexed for 30 s, and kept in the dark for 10 min at 40 °C. After evaporation, 300 μl of water:acetonitrile (75:25, v/v) mixture was added to the vials and vortexed for 30 s. AFM1 standard (c=10 μg/ml) was purchased from Sigma Aldrich (Buchs, Switzerland). The HPLC analysis was performed on an Agilent 1260 (Agilent Technologies Inc., USA) system with fluorescence detector and Agilent Hypersil ODS C18, 4.6 x 100 mm, 5 μm column. The mobile phase consisted of an isocratic mixture of water:acetonitrile (75:25, v/v) at flow rate of 1.0 ml/min. Twenty microliters of standards and samples were injected into the HPLC column. The fluorescence detector was set to an excitation and emission wavelengths of 360 and 423 nm, respectively. The limit of quantification (LOQ) was equivalent to 0.010 μg/kg of AFM1 in a sample. All samples were analyzed in triplicate.

Table 1. Key steps in the production of hard cheese from cow's milk (Popović-Vranješ et al., 2013)

Technological operations	Technological indicators
Milk reception and cooling	Milk fat: 3.80–3.84%; Proteins: 2.93–3.19%
Milk pasteurization	72°C/15 sec
Milk cooling	32°C
Milk in cheese vat	32°C
Adding of milk cultures and stirring	Mesophilic and thermophilic cultures
Adding of $CaCl_2$ and stirring	0.02%
Adding of rennet and stirring	16 g/1.000 l of milk
Coagulation	30–40 min
Cutting curd horizontally and vertically into cubes and leaving the curd to separate whey	10 min
Curd stirring	10 min
Hot water adding	42°C; 30%
Stirring	35–40 min
Milk heating up	42°C; 15–20 min
Termination of stirring and curd precipitation	10–15 min
Cheese lump is roughly cut into cubes that are transferred on the table for molding	
Molding	20–30 min
Pressing	2 bar/20 min; 4 bar/30 min; 6 bar/45 min
Cheese cooling	
Salting	salt 19–21%; pH 5.20; 11–13°C; 2 days
Cheese drying	1 day /12°C
Maturation chamber I	12–14°C; RH 83–87%
Maturation chamber II	10–13°C; RH 85–90%
Maturation time	60–66 days
Cheese washing	water temperature 8–9 °C
Cheese in drying chamber	9–14°C/2 days
Labeling and packaging into cardboard boxes	Shelf life: 9 months or more

RH – relative humidity

RESULTS AND DISCUSSION

Milk samples included two samples (No. 1 and 2; Table 2) containing AFM1 above the EU maximum level (0.077±0.009 and 0.118±0.008 μg/kg) and two samples below the maximum level of 0.05 μg/kg (0.021±0.002 and 0.034±0.004 μg/kg). Regarding AFM1 transfer into cheese from the milk samples containing AFM1 above the EU maximum level, the rate was approximately 4 times higher than the AFM1 level found in the milk (383% and 410%). On the other hand, in the cheese samples (No. 3 and 4; Table 2) made from milk containing AFM1 below the EU maximum level, almost 10-fold levels of AFM1 content were found (transfer rates of 934% and 961%). This could indicate that there might be a relationship between AFM1 level in milk and its transfer rate into cheese, and probably into whey.

Table 2. AFM1 content in milk and its transfer into cheese and whey.

Sample No.	Milk	Cheese		Whey	
	AFM1 (μg/kg)	AFM1 (μg/kg)	Transfer rate	AFM1 (μg/kg)	Transfer rate
1	0.077±0.009	0.293±0.017	383%	0.060±0.003	78%
2	0.118±0.008	0.484±0.008	410%	0.088±0.005	74%
3	0.021±0.002	0.192±0.015	934%	0.014±0.002	68%
4	0.034±0.004	0.322±0.037	961%	0.019±0.001	57%

As expected, in all whey samples AFM1 levels were below the levels found in the milk samples. Transfer rates in the whey samples were 78%, 74%, 68% and 57%.

In the literature, there are studies about the degree of AF transfer into milk products, but it is difficult to make a unique conclusion. There is high standard deviation of AFM1 results in milk products, and there is also large difference between its concentration in milk and milk products (Jakšić et al. 2017).

Popović-Vranješ et al. (2014) found similar values of AFM1 in different types of cheese, and these values were 2–3 times higher compared to the AFM1 levels in the milk, while lower transfer rates were obtained for whey (23.6% to 38.5%). The authors did not comment on the differences in AFM1 transfer rates for various AFM1 levels in the milk samples. Manetta et al. (2009) found that in the production of long maturing cheese, AFM1 levels increased both in curd (3-fold) and in long maturing cheese (4.5-fold), while AFM1 occurrence in whey decreased by 40%, which is in accordance with our results. Cavallarin et al. (2014) used a different approach in quantifying AFM1 transfer rate into cheese and whey. The authors did not express AFM1 content as mass ratio but as total amount in the product. In this manner they concluded that in the production of hard cheese about 30-35% AFM1 from milk was transferred into cheese, while the remaining quantity was discarded into whey. Similar methodology was used by Iha et al. (2013) on soft cheeses. It was found that AFM1 in cheese was 1.9-fold higher, while in whey it was 0.6-fold lower than in milk. Furthermore, considering total amount of AFM1 in the products, they found that similar amounts were transferred into cheese and whey (approximately 30–40% in each), while the remaining quantity was considered as decrease.

CONCLUSION

These results may indicate the possibility that the AFM1 transfer rate from milk into cheese depends on the content of this toxin in milk. More extensive research is needed in order to determine the relationship between these two elements along with the influence of technological conditions of cheese production, and moreover, to provide the recommendations for ML of AFM1 in hard cheese.

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STEPEN PRELASKA AFLATOKSINA M1 IZ MLEKA U SIR I SURUTKU TOKOM PROIZVODNJE TVRDOG SIRA

Izvod: Cilja ovog rada je da se ispita stepen prelaska aflatoksina M1 (AFM1) iz prirodno kontaminiranog sirovog kravljeg mleka u sir i surutku, tokom proizvodnje Livanjskog sira (tip tvrdog sira). Uzorci sirovog kravljeg mleka su prikupljeni sa 4 poljoprivredna gazdinstva sa teritorije Srbije. Pomenuti uzorci su analizirani na sadržaj AFM1, a potom su korišćeni za proizvodnju tvrdog sira. Napravljena su 4 uzorka tvrdog sira, pri čemu su sakupljeni i uzorci pripadajuće surutke. Odabir uzoraka mleka je vršen na način da su dva uzorka sadržavala AFM1 iznad maksimalno dozvoljenih količina (MDK) propisanih regulativnom Evropske Unije (0,077±0,009 i 0,118±0,008 µg/kg), a da su druga dva sadržavala AFM1 ispod pomenute MDK (0,021±0,002 i 0,034±0,004 µg/kg). Kada je u pitanju stepen prelaska AFM1 iz mleka sa sadržajem ovog mikotoksina preko MDK, ostvarene su četvorostruke vrednosti sadržaja AFM1 u siru u odnosu na mleko. S druge strane, u siru proizvedenom od mleka sa sadržajem AFM1 ispod MDK, dobijene su gotovo desetostruke vrednosti u odnosu na mleko od kojeg je napravljen. Kao što se i očekivalo, vrednosti AFM1 u svim uzorcima surutke su bile niže u odnosu na vrednosti iz sirovog mleka (stepen prelaska od 78%,74%, 68% i 57%). Razlike u stepenu prelaska AFM1 između uzoraka sira napravljenih od mleka sa različitim stepenom kontaminacije mogu da ukažu na to da postoji verovatnoća da stepen prelaska AFM1 iz mleka u sir može zavisiti od sadržaja ovog toksina u mleku.

Ključne reči: ***aflatoksin M1, stepen prelaska, mleko, tvrdi sir, surutka, HPLC-FLD***

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