Original paper

TRANSFER OF AFLATOXIN M1 FROM THE CONTAMINATED MILK INTO CHEESE AND WHEY

Popović-Vranješ A.[1], Ješić G.[1], Lopičić-Vasić T.[1], Grubješić G.[1], Kralj A.[2]*

[1]Faculty of Agriculture, University of Novi Sad, Republic of Serbia;
[2] Faculty of Agriculture, University of Banja Luka, Republic of Srpska
*Corresponding author: anka.popovic@gmail.com

Abstract

Aflatoxin M1 is a major metabolite of aflatoxin B1 which is formed when animals ingest contaminated feed. Aflatoxin B1, when ingested by an animal, is rapidly absorbed in the gastrointestinal tract and transformed into a metabolite aflatoxin M1, appearing in the blood after 15 minutes and then secreted in the milk from the mammary gland. Aflatoxin B1 shows hepatotoxic and carcinogenic effects and aflatoxin M1 has a distinct genotoxicity, carcinogenicity and cytotoxicity. The resistance to heat treatment and mild acidic conditions used in the production of cheese or other dairy products (such as, for example, yogurt, cream, butter, and ice cream) has been accounted for the contamination of such products. It is known that aflatoxin M1 is bound to milk proteins, mainly casein, and therefore the toxin is more concentrated in the cheese than in the milk used in cheese production. In practice, aflatoxin M1 can be found in dairy products at levels that are 2-5 times higher than in milk. As a result of the binding of aflatoxin M1 to milk proteins the toxin is distributed more in curd than in whey.

Control of samples of milk from the Vojvodina market was conducted during March and April of 2014, in the Dairy Laboratory and Laboratory for the Analysis of Animal Feed and Animal Products, the Department of Animal Science, Faculty of Agriculture, Novi Sad. The method used was HPLC with fluorescence detection with pre-treatment of milk on immunoaffinity columns. The analysis of milk was performed on milk of 12 dairies whose products can be found on the Vojvodina market. The manufacturer with the highest levels of aflatoxin M1 in milk was chosen and a semi-hard cheese was made of such milk.

The results showed the expected increase of concentration of aflatoxin M1 in cheese and whey. Since Serbia has no regulation that determines the minimum allowable level of aflatoxin M1 in cheese and other dairy products, the results were compared with acceptable values for cheese in some European countries.

Keywords: *aflatoxin M1, cheese, HPLC, milk, whey*

Introduction

Aflatoxins (AFS) are highly toxic secondary metabolites produced by Aspergillus, Penicillium and Rhizopus strains in cultures of herbal products, of which aflatoxin B1 (AFB1) is the most representative (Goldblatt, 1969; Rubio et al., 2011). If AFB1 is present in animal feed during lactation the animals excrete aflatoxin M1 (AFM1) in their milk (Allcroft and Carnaghan, 1963; Cupid et al., 2004; Battacone et al., 2005). It is a toxin classified by the International Agency for Research on Cancer as a possible cause of cancer (IARC, 2002).

Given AFM1 has an impact on the health of consumers through insertion of milk, according to the Codex Alimentarius Commission, the maximum determined recommended concentration is 500 ng kg-1 (Codex Alimentarius Commission, 2001) although the limit (50 ng kg-1) for raw milk, milk products and heat-treated milk is given by regulations in the European Union (EU Commission Regulation, 2003). As for the cheese, only in a few countries (Netherlands, Switzerland, Austria, Turkey and Italy) there are prescribed boundaries within the range of 200-450 ng kg-1 for the presence of AFM1. In Serbia, there are no regulations to control the presence of AFM1 in dairy products.

When the cheese is produced from AFM1-contaminated milk, this toxin will probably be present in the cheese made from that milk. This could be explained by the fact that AFM1 is bound to casein (Applebaum et al., 1982; Battacone et al., 2005) and increase dry matter content (Deveci, 2007).

The affinity of AFM1 was tested not only for this protein, but also for others, such as whey proteins (Mendoca and Venancio, 2005). Therefore, it is necessary to note whether AFM1 is present in the final products such as cheese, and its concentration therein is approximately 2.1 to 4.5 times higher than in the original milk used for cheese production, depending on the cheese type (Van Egmond, 1983; Viseman and Marth, 1983; Blanco et al., 1988; Deveci, 2007; Manetta et al., 2009). Yet in a variety of products, such as yogurt, level of AFM1 is stable and does not affect the production of yogurt (Blanko et al., 1993). Accordingly, AFM1 may also be present in other dairy products such as whey and products obtained from whey.

AFM1 transfer in the amount of 40-60% was recorded in the whey in relation to the AFM1 which is present in milk (Govaris et al., 2001; Oruc et al., 2006; Deveci, 2007; Kamkar et al., 2008; Manetta et al., 2009). However, this scope can be higher, between 70% and 74% (Battacone et al., 2005), or much lower, between 17% and 27% (Lopez et al., 2001). This wide range of AFM1 distribution in cheese production could be due to different factors; for example, techniques used prior to HPLC analysis (Battacone et al., 2005; Kamkar et al., 2008), which is associated with the size of the sample or solvent extraction. Characteristics of the production process, depending on the type of cheese, whether it is hard (Brackett and Marth, 1982; Blanco et al., 1988; Manetta et al., 2009) or soft cheese (Viseman and Marth, 1983; Govaris et al., 2001; Oruc et al., 2006) also may affect the distribution of AFM1. In addition, the type of contamination of milk should also be taken into account, because when milk is naturally contaminated (Virdiset et al., 2008), AFM1 values are usually lower than in the artificially contaminated milk (Deveci, 2007).

This paper aims to investigate the presence of AFM1 in milk and its distribution in milk products that can be found on the market of AP Vojvodina.

Materials and methods

The analysis of chemical composition (fat, protein, lactose and total solids and somatic cells) was done for all samples of milk from the market on the CombiFoss FT + machine (FossElectric, Hillerød, Denmark) calibrated using the certified standards which combine MilkoScanTM FT + (infrared spectrometric analysis), previously calibrated for cow's milk, with FossomaticTM FT + (counting somatic cells based on flow cytometry), in Dairy Laboratory at the Department of Animal Science, Faculty of Agriculture in Novi Sad.

Detection of presence of AFM1 in the milk samples was performed by standard HPLC high performance liquid chromatography in the Laboratory for the Analysis of Animal Feed and Animal Products to the Department of Animal Science, Faculty of Agriculture in Novi Sad.

Milk samples, in which the highest level of AFM1 was detected, were used for production of semi-hard cheese. Cheese production took place using the standard recipe for the production of semi-hard cheese in Dairy Laboratory at the Faculty of Agriculture in Novi Sad. Cheese was made from milk from 6 diaries where AFM1 levels were the highest and where three cheeses per diary were made. Process for the production of cheese was carried out as follows: contaminated milk heated at a temperature of 35 °C, $CaCl_2$, cultures and rennet are added. The renneting of milk is followed by further course of standard technological operations and manipulations such as coagulation, curd draining and pressing to obtain the product. In the production of cheese the whey is separated from the curd.

The obtained cheese and whey were further analyzed for the presence of AFM1, using standard high performance liquid chromatography - HPLC, Agilent Technologies 1260 Infinity LC system, a Hypersil ODS column, with FLD detector and a mobile phase of acetonitrile-water (v/v, 25:75).

Testing: Cuvette for centrifuge with volume of 50 cm^3 was filled with a sample of milk to the top and heated up to a temperature between 37-40 °C, about 30 minutes. Then the milk sample is shaken for fat to disperse, and cuvettes with the samples are centrifuged at 3000 rotations/min for 10 minutes. The layer of fat was removed, and the sample is filtered through a quantitative filter paper (black belt). After that, 50 cm^3 of the filtrate is heated at 37 °C for about 10 minutes. The sample was passed through immunoaffinity column by free fall. The column is washed with 20 cm^3 of deionized water and eluted with approximately 4 cm^3 of acetonitrile. The contents of the tube are evaporated in the thermoblock at 60 °C in a stream of nitrogen. Then, add 200 µl of n-hexane and 200 µl trifluoroacetic acid and stir the vortex for about 1 minute. The contents of the tubes were then incubated in the thermoblock 40 °C exactly 10 minutes, after which a stream of nitrogen at the same temperature evaporate it to dryness. The residue was then dissolved in 300 µl mobile phase by vortex mixing for about 1 minute and transferred to an HPLC vial.

Chromatography was performed on a C-18 column size 4.6 x 100 mm, 5 mm, at a flow rate of the mobile phase (acetonitrile-water (v/v, 25:75)) of 1 ml/min and a column temperature of 40 °C. Injection volume is 20 µl. The detection is performed using FLD detector at the following wavelengths: 365 nm excitation, 440 nm emission.

For a proper interpretation of the results, analysis of variance (ANOVA) and Tukey's post-hoc test were used for significance between the observed characteristics, while the coefficient of correlation was determined using the Pearson test, in the Statistica 12 software.

Results and discussion

Table 1 shows the results of the analysis of the samples of milk and dairy products that were monitored on the market of AP Vojvodina for the presence of AFM1.

Table 1. *Results of analysis of milk and milk products from AP Vojvodina market on AFM1*

Dairy No.	Product	Fat%	Aflatoxin M1 (ppb) µg/kg
1.	Pasteurized milk	2.8	0.048
1.	Yoghurt	2.8	0.000
2.	Yoghurt	2.8	0.209*
2.	Pasteurized milk	2.8	0.150*
2.	Trapist cheese	min 45%	0.478*
2.	Pasteurized milk	2.8	0.124*
2.	Pasteurized milk	2.8	0.124*
3.	Pasteurized milk	2.8	0.075*
3.	Yoghurt	2.8	0.035
3.	Yoghurt	2.8	0.000
4.	Pasteurized milk	2.8	0.021
4.	Yoghurt	2.8	0.039
4.	Cheese made using ultrafiltration	min 45%	0.145*
5.	Pasteurized milk	2.8	0.127*
5.	Yoghurt	2.8	0.094*
5.	Cheese made using ultrafiltration	min 45%	0.281*
6.	Sour milk	2.8	0.028
6.	Pasteurized milk	2.8	0.070*
6.	Trapist cheese	min 45%	0.233*
7.	Sour milk	2.8	0.074*
8.	Pasteurized milk	2.8	0.019
9	Pasteurized milk	2.8	0.113*
10	Pasteurized milk	2.8	0.046
11	Pasteurized milk	2.8	0.070*
12	Pasteurized milk	2.8	0.000

* Levels of AM1 exceeding maximum allowed concentration

Numbers from 1-12 represent dairies that sell milk and milk products on the market of AP Vojvodina and whose products are analyzed.

Based on the results of analysis of samples of pasteurized milk, milk with a high concentration of AFM1 from the dairies: 2, 3, 5, 9, 10, 11, fermented cheese and whey, which are further analyzed for the presence of AMF1 and thereby analyzed for the transfer of toxins from milk into milk products.

Table 2. *Results of analysis of cheese and whey from dairies No. 2, 3, 5, 9, 10, 11*

Dairy No.	Product	Fat%	Aflatoxin M1 (ppb) µg/kg
2.	Pasteurized milk	2.8%	0.124*
2.	Cheese from pasteurized milk, dairy No. 2		0.296
2.	Whey separated from curd of cheese made from pasteurized milk, dairy No. 2		0.047
3.	Pasteurized milk	2.8%	0.075*
3.	Cheese from pasteurized milk, dairy No. 3		0.160
3.	Whey separated from curd of cheese made from pasteurized milk, dairy No. 3		0.019
5.	Pasteurized milk	2.8%	0.127*
5.	Cheese from pasteurized milk, dairy No. 5		0.303
5.	Whey separated from curd of cheese made from pasteurized milk, dairy No. 5		0.030
9.	Pasteurized milk	2.8%	0.113*
9.	Cheese from pasteurized milk, dairy No. 9		0.339
9.	Whey separated from curd of cheese made from pasteurized milk, dairy No. 9		0.035
10.	Pasteurized milk	2.8%	0.046
10.	Cheese from pasteurized milk, dairy No. 10		0.181
10.	Whey separated from curd of cheese made from pasteurized milk, dairy No. 10		0.018
11.	Pasteurized milk	2.8%	0.070*
11.	Cheese from pasteurized milk, dairy No. 11		0.281
11.	Whey separated from curd of cheese made from pasteurized milk, dairy No. 11		0.027

* Levels of AM1 exceeding maximum allowed concentration

Based on the obtained results, we can perceive that out of 25 analyzed samples of milk and milk products from 12 manufacturers placing their products on the market in the territory of AP Vojvodina the AFM1 was detected in 23 samples. Of the total number of 14 samples of pasteurized milk samples 10 samples have been contaminated with AMF1 in concentration above 0.05 mg/kg, which is specified as the maximum permissible concentration according to the current Regulation on amendments to the Regulation on the maximum level of residues for protection found in food products and feed and the animal food and feed which are determined as the maximum allowable amount of residues found in plant nutrition (Official Gazette of RS, 2014). Seven fermented milk products were analyzed, and in six samples the presence of aflatoxin M1 is evident, while three samples have levels of aflatoxin M1 above 0.05 mg/kg. Further, in the four analyzed samples of cheese, all four had high levels of aflatoxin M1, although in Serbia there are no legal regulations that restrict the presence of toxins in the cheese and in other milk products where their presence is evident and sometimes have several times higher concentration than in milk from which it has been produced.

At the global level, the maximum allowed level of aflatoxin M1 in cheese differs as it is presented in Table 3.

Table 3. *The maximum permitted level of aflatoxin AFM1 in cheese in different countries (Anfossi et al., 2011)*

Country	**Maximum permitted level (ng/kg)**
Argentina	500
Austria	250
Switzerland	250
Egypt	0
Honduras	250
Italy	250 (450*)
Romania	0
Netherlands	200
Turkey	250

* Level of AFM1 in hard cheeses

Most countries have set a limit and decided for the maximum permissible level of 250 ng/kg, which corresponds to the assumption that the cheese is made from milk which is in accordance with the regulations (ie. contaminated at levels below 50 ng/kg) and the concentration of AFM1 can grow up to 5-times due to dehydration. However, some countries have opted for a strategy of zero tolerance (Romania and Egypt) to ensure maximum protection of the health of consumers at the expense of producers of milk and cheese. By contrast, in 2004, Italy raised the limit of AFM1 in hard cheese to 450 ng/kg to protect the production of Parmesan cheese which in that year had big problems due to a high contamination of animal feed with AFB1.

In all six of the analyzed samples of cheese made in the Laboratory of contaminated milk with a high concentration of AFM, there were detected two to three times higher levels than the concentration of AFM1 in milk.

In the samples of cheese made from milk of dairy No. 2, the concentration of AFM1 doubled and was 0.296 mg/kg compared to the concentration of AFM1 in milk of 0.124 mg/kg ppb. The whey, which is obtained during the production of cheese from contaminated milk, was also analyzed and the presence of AFM1 was detected at the concentration of 0.047 mg/kg.

In the samples of cheese made from milk produced in dairies No. 3 and 5, the concentration of AFM1 detected was 2.13 and 2.3 times higher in relation to the milk from which they were made and amounts to 0.160 mg/kg and 0.303 mg/kg. In the sample of cheese made from milk of dairy No. 9, AFM1 concentration was three times higher than in milk from which it was made and amounts to 0.339 mg/kg.

In the cheese sample from milk of dairy No. 10, the increase of AFM1 concentration was 0.181 mg/kg compared to the concentration found in milk which was used for manufacturing the same cheese being 0.046 mg/kg. Whey, which is obtained during the production of the cheese samples from contaminated milk had AFM1 at the concentration of 0.018 mg/kg. It has been noted that the concentration in sample cheese from 11th dairy is three times higher than in milk from which it was made - AFM1 concentration was 0.07 mg/kg, and in cheese from that milk was 0.281 mg/kg.

Based on the results of analysis of whey samples it can be seen the transfer of AFM1 from milk to whey in the amount of 23.6% to 38.5% and ranges from 0.018 mg/kg to 0.046 mg/kg in the analyzed samples of whey.

Software for statistical analysis of the data Statistica 12 shows the correlation between milk and cheese as well as the correlation between milk and whey and based on the results observed in both cases there is a high correlation between the studied parameters ($r = 0.76$ and $r = 0.80$) with statistically significant difference $p \leq 0.01$.

Conclusion

Based on the results obtained in this study it is evident that 2-3 times increase in the concentration of AFM1 in cheese is in relation to contaminated milk from which it is produced. The above facts should be the guidelines to initiate the establishment of regulations concerning the allowable limit of aflatoxin M1 in cheese and other dairy products in the Republic of Serbia.

The results of research in this paper also point to failures and lack of control of animal feed, which is indirectly reflected in the distortion - in this case the quality of milk and milk products that are used in human nutrition. These results are worrying because we must take into account that the effect of AFM1 is toxic and carcinogenic to human health.

This is the reason why we should systematically take all measures for the production of healthy food for the animals which would provide production of milk and milk products within the permissible values for the member states of the European Union.

References

1. Allcroft, R., Carnaghan, R.B.A. 1963. Groundnut toxicity: an examination for toxin in human food products from animal fed toxic groundnut meal. Veterinary Research 75, 259-263.
2. Anfossi, L., Baggiani, C., Giovannoli, C., Giraudi, G. 2011. Occurrence of Aflatoxin M1 in Dairy Products, Aflatoxins - Detection, Measurement and Control, Dr Irineo Torres-Pacheco (Ed.), ISBN:978-953-307-711-6, InTech
3. Applebaum, R., Brackett, R.E., Wiseman, D.W., Marth, E.H. 1982. Aflatoxin: toxicity to dairy cattle occurrence in milk and milk products. Journal of Food Protection 45, 752-777.
4. Battacone, G., Nudda, A., Palomba, M., Pascale, M.,Nicolussi, P., Pulina, G. 2005. Transfer of aflatoxin B1 from feed to milk and from milk to curd and whey in dairy sheep fed artificially contaminated concentrates. Journal of Dairy Science 88, 3063-3069.
5. Blanco, J.L.,Carrión, B.A., Liria, N., Díaz, S., García, M.E., Domínguez, L., Suárez, G. 1993. Behaviour of aflatoxins during manufacture and storage of yoghurt. Milchwissenschaft 48, 385-387.
6. Blanco, J.L., Domínguez, L., Gómez-Lucía, E., Garayzabal, J.F.F., Goyache, J., Suárez, G. 1988. Behaviour of aflatoxin during manufacture, ripening and storage of Manchego-type cheese. Journal of Food Science 53, 1373-1376.

7. Brackett, R.E., Marth, E.H. 1982. Fate of aflatoxin M1 in Cheddar cheese and in process cheese spread. Journal of Food Protection 45, 549-552.
8. Codex Alimentarius Commission 2001. Comments submitted on the draft maximum level for aflatoxin M1 in milk. Codex committee on food additives and contamination 33rd session, The Hague, The Netherlands.
9. Cupid, B.C., Lightfoot, T.J., Russel, D., Gant, S.J.,Turner, P.C., Dingley, K.H. 2004. The formation of macromolecular adducts in rat and human at dietary levels of exposure. Food and Chemical Toxicology AFB1 42, 559-569.
10. Deveci, O. 2007. Changes in the concentration of aflatoxin M1 during manufacture and storage of White Picked cheese. Food Control 18, 1103-1107.
11. European Commission, Commission Regulation No.2174/2003 of 12 December 2003, amending Regulation (EC) No 466/2001 as regards aflatoxins. Official Journal of the European Community L326, 12-15.
12. Goldblatt, L.A. 1969. Aflatoxin. Scientific Background, Control and Implications. L.A. Goldblatt (Ed.),Academic Press, New York and London, pp 1-54.
13. Govaris, A., Roussi, V., Koidis, P.A., Botsoglou, N.A. 2001. Distribution and stability of aflatoxin M1 during processing, ripening and storage of Telemes cheese. Food Additives and Contaminants 18, 437-443.
14. International Agency for Research on Cancer (IARC) 2002. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monographs Vol.82, Lyon, France.
15. Kamkar, A., Karim, G., Shojaee Aliabadi, F., Khaksar, R. 2008. Fate of aflatoxin M1 in Iranian white cheese processing. Food and Chemical Toxicology 46, 2236-2238.
16. López, C., Ramos, L., Ramadán, S., Bulacio, L., Perez, J. 2001. Distribution of aflatoxin M1 in cheese obtained from milk artificially contaminated. International Journal of Food Microbiology 64, 211-215.
17. Manetta, A.C., Giammarco, M., Di Giuseppe, L., Fusaro, I., Gramenzi, A., Formigoni, A., 19. Mendoça, C., Venâncio, A. 2005. Fate of aflatoxin M1 in cheese whey processing. Journal of the Science of Food and Agriculture 85, 2067-2070.
18. Official Gazette of RS 72/14, 2014. Regulation on the maximum level of residues for protection found in food products and feed and the animal food and feed which are determined the maximum allowable amount of residues found in plant nutrition
19. Oruc, H.H., Cibik, R., Yilmaz, E., Kalkanli, O. 2006. Distribution and stability of aflatoxin M1 during processing and ripening of traditional white pickled cheese. Food Additives and Contaminants 23, 190-195.
20. Rubio, R., Moya, V.J., Berruga, M.I., Molina, M.P. 2011. Aflatoxin M1 in the intermediate dairy products from Manchego cheese production: distribution and stability; in the intermediate dairy products, Mljekarstvo 61 (4), 283-290 283.
21. Van Egmond, H.P. 1983. Mycotoxins in dairy products.Food Chemistry 11, 289-307.
22. Vignola G., Lambertini L. 2009. Distribution of aflatoxin M1 during Grana Padano cheese production from naturally contaminated milk. Food Chemistry 113, 595-599.
23. Virdis, S., Corgiolu, G., Scarano, C., Pilo, A.L., De Santis, E.P.L. 2008. Occurrence of Aflatoxin M1 in tankbulk goat milk and ripened goat cheese. Food Control 19, 44-49.
24. Wiseman, D.W., Marth, E.H. 1983. Behaviour of aflatoxin M1 during manufacture and storage of Queso Blanco and Backers' cheese. Journal of Food Protection 46, 910-913.