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Vesna V. Jankovic[1]
Jelena B. Vukojevic[2]
Brankica M. Lakicevic[1]
Radmila R. Mitrovic[1]
Dejan I. Vukovic[3]

[1] Institute of Meat Hygiene and Technology, Kaćanskog 13, 11000 Belgrade, Serbia
[2] Institute of Botany, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia
[3] Veterinary station, PKB, Padinska skela 23, 11000 Belgrade, Serbia

PRESENCE OF MOULDS AND AFLATOXIN M1 IN MILK

ABSTRACT: Aflatoxin M1 (AFM1) appears in milk or dairy products as a direct result of the cattle's ingestion of feed contaminated with aflatoxin B1 (AFB1).

This study comprises mycological and mycotoxicological investigations of 23 milk samples (raw, infant food, pasteurized, whey and yoghurt).

The mycological testing showed dominant presence of genus *Geotrichum*. *G. candidum* was found in 9 samples, with the highest contamination in the raw milk samples.

The contamination level of AM1 is defined by using direct competitive enzyme-linked immunosorbent assay (ELISA). AFM1 was found in 9 samples. AFM1 levels were lower than the recommended limits.

However, as AFM1 is considered a probable human carcinogen (2B type), it is necessary to achieve a low level of AFM1 in milk.

Therefore, cows' feed samples from various cowsheds are supposed to be evaluated routinely for aflatoxin, and kept away from fungal contamination as much as possible.

KEYWORDS: milk, moulds, aflatoxin M1, enzyme immunoassay (ELISA)

INTRODUCTION

Aflatoxins are extremely toxic metabolites produced by the common fungi *Aspergillus flavus, A. parasiticus* and *A. nomius* (B a s k a y a et al., 2006). The most important are aflatoxin B_1(AB_1), G(AG_1) and their dihydro derivatives AB_2 and AG_2. Given that they are some of the most powerful mutagens and carcinogens, and on the basis of all-round scientific knowledge, in 1987 IARC classified them into Group 1 of human carcinogens.

Aflatoxin M1 appears in milk and milk products as a direct result of the intake of aflatoxin B1 contaminated feed by dairy cows (Van Egmond, 1989). AB1 may occur in various agricultural products that are used as feedstuff ingredients. The amount excreted as AM1, as a percentage of AB1 in feed, is usually 1—3%, but the values as high as 6% were reported by Pitet, 1998. Rothschild (1992) classified L.J. Rothschild , IARC classes AFB1 as class 1 human carcinogen. *Food Chem.* 34 (1992), pp. 62—66. AFM1 as 2B (probable carcinogen) human carcinogens. Lafont, Siriwardana and Lafont (1989) have also observed that AFM1 has high genotoxic activity, although AFM1 has been found to be about 10 times less carcinogenic than AFB1. In 2002, IARC classified AM1 into Group 1.

The risk posed by aflatoxins has been faced differently in different countries. In Europe, the maximum tolerated levels of AFM1 in milk and dairy products were regulated firstly by Regulation CE 2174/2003 (Off. J. Eur. Communities, 2003) that modified Regulation CE 466/2001 (Off. J. Eur. Communities, 2001), and then by Regulation 1881/2006 (Off. J. Eur. Communities, 2006). In accordance with these norms, the product to be screened is milk, in which AFM1 concentration must not exceed 0.05 mg/kg, while dairy products must be obtained using milk conforming to the above AFM1 limits.

In Serbia, maximum tolerated level of AM1 is 0.5 mg/kg (Official Gazette of SRY, No. 11/92).

This study was carried out to evaluate the presence of moulds and the prevalence of milk contamination with AM1.

MATERIALS AND METHODS

Mycological research

The mycological research encompassed determination of the total number of moulds in 1 ml of the tested samples and their identification. Twenty three different samples were tested (raw milk, infant food, whey, yoghurt and pasteurized milk). Seven samples of raw milk were taken from PKB, veterinary station, while the other samples were taken from the market.

Determination of the total number of moulds in 1 ml of milk was conducted in duplicate, according to the standard laboratory procedure.

Two types of culture media were used: Sabouraud-maltose agar (SMA) with the addition of antibiotics (1 ml chloramphenicol per 100ml of medium), and maltose yeast extract agar with 50% of glucose. The isolated species were identified on the basis of investigation of the macromorphological properties of colonies and micromorphological properties of conidial and other structures, and according to the key described by Samson and van Reenen-Hoekstra, 1988.

Mycotoxicological research

The quantitative analyses of AFM1 were performed with the enzyme immunoassay: Tecna — aflatoxin M1. The test kit is sufficient for 96 determinations (including the calibration curve).

The basis of the test is the antigen-antibody reaction. The wells in the microtiter strips are coated with specific antibodies reactive to AFM1. By adding AFM1 standards, or the sample solutions, the antibody binding sites are occupied proportionally to the AFM1 concentration. Any remaining free binding sites are occupied in the next stage by enzyme labeled toxin (enzyme conjugate). Any unbound enzyme conjugates are then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop reagent leads to a colour change from blue to yellow. The measurement is made photometrically at 450 nm (optional reference wavelength 600 nm). The absorption is inversely proportional to the AFM1 concentration in the sample.

RESULTS AND DISCUSSION

Mycological results

Eleven samples were without growth, while raw milk (samples 2 and 5) and infant food (sample 9) had the highest contamination level ($4 \cdot 10^3$/ml). Whey samples showed growth from $1.2 \cdot 10^2$/ml to $2 \cdot 10^2$/ml (Table 1). Comparison of the results pointed out that there were no drastic differences between the numbers of moulds grown on the two culture mediums.

According to the mycological research, genus *Geotrichum* was dominant in 9 out of 23 samples. The remaining moulds were from genus *Aspergillus*. *G. candidum* was isolated in raw milk (samples 1, 2, 3, 5 and 7), infant food (9 and 10), pasteurized milk (17) and yoghurt (sample 22). The level of contamination with *G. candidum* was the smallest in the samples 3, 7, 10, 17 and 22, while the samples 2, 5 and 9 had the highest level of contamination ($4 \cdot 10^3$/ml).

Aspergillus spp. were dominant in whey samples (14, 15 and 16), with the level of presence ranging from 1.2/ml — $2 \cdot 10^2$/ml.

The results are similar to the results of Torkar et al. (2007), who reported that the genera most frequently isolated from raw milk belonged to genera *Geotrichum* (51.5% of strains) and *Aspergillus* (33.8% of strains).

The presence of toxic metabolite AM1

Out of a total of 23 samples of various milk (raw, infant food, pasteurized, whey and yoghurt), 9 samples contained AFM1 in the quantities ranging from 0.007 to 0.250 μg/kg . AFM1 was not detected in any of the 6 samples

of pasteurized milk. The presence of AFM1 was very low in infant food (sample 9) and yoghurt (sample 20). All detected concentrations were practically below the maximum tolerated levels of AM1 — 0.5 μg/kg (Official Gazette of SRY, No. 11/92). Only three samples (raw milk) showed concentrations higher than the maximum tolerance limit of 0.05 μg/kg accepted by the EU regulations (samples of raw milk — 1, 2 and 5).

The percentages of absorbance obtained by the competitive enzyme immunoassay with the calibration curve (Table 1) allow the calculation of AFM1 concentration in mg/kg in the positive samples, for each type of milk.

Tab. 1 — Concentration of AM1 and total number of moulds in 1ml of milk

Sample	Aflatoxin M1 ($\mu g \cdot kg^{-1}$)	Total No. (moulds/ml)
Raw milk 1	0.250	$3 \cdot 10^3$
Raw milk 2	0.120	$4 \cdot 10^3$
Raw milk 3	0	20
Raw milk 4	0	0
Raw milk 5	0.200	$4 \cdot 10^3$
Raw milk 6	0	0
Raw milk 7	0.02	30
Infant food 8	0	0
Infant food 9	0.02	$4 \cdot 10^3$
Infant food 10	0	20
Pasteurized milk 11	0	0
pasteurized milk 12	0	0
pasteurized milk 13	0	0
Whey 14	0.042	$2 \cdot 10^2$
Whey 15	0.020	$2 \cdot 10^2$
Whey 16	0.008	$1.2 \cdot 10^2$
pasteurized milk 17	0	15
pasteurized milk 18	0	0
pasteurized milk 19	0	0
Yoghurt 20	0.007	0
Yoghurt 21	0	0
Yoghurt 22	0	20
Yoghurt 23	0	0

Considering the evaluation of AM1 by ELISA, in Italy, AFM1 was found in 16.6% of the cheeses tested. 31.3% of these were sheep-goat cheeses, 27.2% were cow cheeses, 16.7% were goat cheeses, and 12.8% sheep cheeses with no significant differences ($p > 0.05$). All the samples of buffalo milk cheese were consistently negative. Overall, the AFM1 values ranged from 50 to 250 ppt. Specifically, the concentrations of AFM1 in goat cheese ranged from 90 to 250 ppt, in cow cheese from 50 to 160 ppt, in sheep cheese from 50 to 215, and in sheep-goat cheese from 55 to 140 ppt (Montagna et al., 2008).

In Argentina, of a total of 77 various types of milk samples, with screening ELISA method, only 18 samples (approximately 23 %) were found to be contaminated with AFM1 at 0.010—0.030 μg/kg. All concentrations were below the maximum tolerated levels of AFM1 in liquid milk and powdered milk (Lopez et al., 2002).

In Iran, aflatoxin M1 was found in 100% of the examined milk samples. 390 samples (62.5%) had contamination less than 45 ng/l of AFM1, 123 samples (19.7%) contained 45—50 ng/l, 94 samples (15.1%) contained 50—80 ng/l, and the remaining 2.7% of the samples contained more than 80 ng/l of AFM1. In general, regardless of the 19.7% of the samples that were in borderline limit (45—50 ng/l), the amount of AFM1 in 17.8% of the samples was higher than the maximum tolerance limit (50 ng/l) accepted by the European Union (Alborzi et al., 2005).

CONCLUSION

In conclusion, considering that for various reasons many regions are obliged to feed dairy animals on stored forage or industrially produced pellets, it is important to reduce the occurrence of toxins (AFB1) in feedstuff and take prophylactic measures to prevent the factors enhancing toxin production. These factors include environmental temperature, humidity, and moisture content of the feed, as well as pH and mechanical damage to the grain affecting mould production.

Moreover, since AFM1 is well known to be mutagenic and carcinogenic, international regulations ensuring a minimal presence of this aflatoxin in cheeses are needed. In fact, by having a common European norm concerning the AFM1 threshold limits for dairy products, it will be possible to guarantee the distribution of safer, healthier foods, particularly in the light of the current norms on internal controls and HACCP (Off. J. Eur. Communities, 2004).

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УТВРЂИВАЊЕ ПРИСУСТВА ПЛЕСНИ И АФЛАТОКСИНА М1 У МЛЕКУ

Весна В. Јанковић[1], Јелена Б. Вукојевић[2], Бранкица М. Лакићевић[1], Радмила Р. Митровић[1], Дејан И. Вуковић[3]

[1] Институт за хигијену и технологију меса, Каћанског 13, Београд, Србија
[2] Институт за ботанику, Биолошки факултет, Таковска 43, Београд, Србија
[3] Ветеринарска станица, ПКБ, Падинска скела 23, Београд, Србија

Резиме

Рад обухвата миколошку и микотоксиколошку анализу 23 узорка млека (свеже кравље млеко, дечја храна, пастеризовано млеко, сурутка и јогурт). Микролошка испитивања обухватала су одређивање укупног броја плесни у 1 ml узорка на две подлоге SMA и MA. Детерминација плесни извршена је на основу микроморфолошких и макроморфолошких особина на основу одговарајућих кључева. Микотоксиколошка испитивања обухватала су утврђивање присуства AM1 коришћењем ELISA теста фирме Тецна.

Детерминацијом плесни утврђена је доминантност врсте *Geotrichum candidum*. Микотоксиколошка испитивања потврдила су здравствену исправност свих узорака према нашем Правилнику.

Неопходно је обезбедити мониторинг сточне хране на присуство AB1, свежег млека на AM1, као и одговарајуће агротехничке мере.

Пројектовано повећање температуре на глобалном нивоу и учесталост и трајање топлотних интервала могу повећати ризик контаминације афлатоксинима и у умереним климатским регионима.