



Detoxification of Aflatoxin M1 in Milk by Lactic Acid Bacteria

Sanaz Seyedjafarri

10.18805/ajdfr.DR-193

ABSTRACT

Background: Aflatoxin M₁ is a highly toxic and carcinogenic compound which is found in milk and milk products and it is a hydroxylated metabolite of Aflatoxin B₁. When the dairy animal digested AFB₁ contaminated feed, it is changed to aflatoxin M₁ and transferred to tissues and milk. Aflatoxin M₁ is less carcinogenic than AFB₁, but it is acutely hepatotoxic as AFB₁. Liver is their main target organ in the body. It has been calculated approximately that 0.3- 6.2% of presented AFB₁ in animal feed transferred as aflatoxin M₁ in milk. Occurrence of AFM1 in milk and milk products is a big concern. Therefore, several countries have standardized the maximum levels of AFM1 in milk and milk products.

Methods: In this experiment, the ability of yoghurt bacteria to degrade AFM1 levels in milk and yoghurt were analysed. The starter culture of yoghurt contains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (1:1). The experiment is carried out in Glasgow Caledonian University, department of life science in 2010.

Result: These bacteria showed the higher binding ability between 90- 100% in milk samples whereas no considerable reduction was observed in yoghurt samples. In some yoghurt samples, an increase of AFM1 level was detected but in overall, concentration of AFM1 was stable in yoghurt. Also, the fat content of milk and yoghurt did not have any negative or positive effect on the concentration of AFM1 in milk and yoghurt.

Key words: Aflatoxin, Lactic acid bacteria, Milk, Yogurt.

INTRODUCTION

Mycotoxins are secondary metabolites of mycelia structure of filamentous or particularly moulds. Mycotoxins are low molecular organic compounds. They are synthesized at the end period of the logarithmic growth phase. Mycotoxins have no biochemical significance in growth and development of fungi (Laciakova *et al.*, 2008). They exert toxic which have harmful effects on humans and animals. Mycotoxins can enter either human foods or animal feeds and cause mycotoxicoses. The severity of toxicity depends on the type of mycotoxins. Aflatoxins (AF), ochratoxins (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), ergots and some unidentified mycotoxins are classified as various types of mycotoxins. These toxins cause economic losses in the worldwide in animal health, human health and agricultural products (Manetta *et al.*, 2005). Mycotoxins are produced under the specific humidity, temperature, PH, availability of essential nutrients and competitive growth of other microorganisms (Celik *et al.*, 2005). To avoid of presence or growth of mycotoxins, appropriate storage of foods and feeds is important.

The health hazards of these toxic metabolites to animals (Govari *et al.*, 2002) and humans (Fallah *et al.*, 2009) have been reconsidered in past few years. The WHO-International Agency of cancer research (WHO, 2017) assessed the carcinogenic effects of AF, OT, ZEN, F and trichothecenes and classified them in three groups. First group was aflatoxins which were known as carcinogenic to humans and animals. OT and F were categorized in second group as possible carcinogenic compounds while trichothecenes and ZEN were not recognized as human carcinogenic (Group 3).

Department of Life Science, Glasgow Caledonian University, UK.

Corresponding Author: Sanaz Seyedjafarri, Department of Life Science, Glasgow Caledonian University, UK.

Email: sseyedjafarri2020@gmail.com

How to cite this article: Seyedjafarri, S. (2021). Detoxification of Aflatoxin M1 in Milk by Lactic Acid Bacteria. Asian Journal of Dairy and Food Research. 40(1): 30-34. DOI: 10.18805/ajdfr.DR-193.

Submitted: 01-09-2020 **Accepted:** 28-12-2020 **Online:** 12-03-2021

Over 300 mycotoxins have been isolated and characterized (Haskard *et al.*, 2001). These toxic metabolites are produced in post-harvest crops and during storage or plant growth. Digestion of these compounds by animal or human cause harmful and destructive effects on animal and humans. Mycotoxins can reduce the growth rate of young animals, impair immunologic responsiveness and interfere with native mechanism of resistance and make animal more susceptible to infection (Hussein and Barsel, 2001). Aflatoxins are heat resistant and are not destroyed by heating treatments such as pasteurisation or sterilization.

Higher concentration of toxicity causes death related to acute kidney and liver damage (Huwig *et al.*, 2001).

Aflatoxins are one of the main groups of mycotoxins. They are highly toxic, carcinogenic, mutagenic, teratogenic compounds which are secondary metabolites of fungi. They are produced by three strains of fungi *Aspergillus flavus*, *A. parasiticus* and *A. nominus*. These fungi are ubiquitous but the peanut meal, maize and cottonseed meal are main sources in feeds. *A. flavus* likes an aerial environment while *Aspergillus. parasiticus* is better adapted to soil environment. Aflatoxins have been found in many animal feeds and foods

(Egmund, 1983). Among 18 types of aflatoxins, the most frequently occurring ones in fungi cultures are aflatoxin B₁, B₂, G₁ and G₂. The best temperature for aflatoxins growth is between 12°C and 40°C (Philips, 1999) and the required water activity is between 0.95- 0.99 (Christou *et al.*, 1985). In 1960, a new disease was reported in poultry farm in England and that was termed "Turkey X disease". Careful investigation on this outbreak showed the toxicity of peanuts meal which turkeys were fed. After intensive speculation in 1960 regarding the nature of the toxin, the fungal source of toxin was proved. The toxin-producing fungal was identified as *Aspergillus flavus* and the toxin was named "Aflatoxin".

MATERIALS AND METHODS

The experiments are carried out in Glasgow Caledonian University Laboratory February-June 2010 wherein there are:

10.4 gr MRS broth powder (DeMan-Rogosa-Sharpe) was dissolved in 200ml distilled water.

12.4 gr MRS agar powder (DeMan- Rogosa- Sharepe) was dissolved in 200ml distilled water.

10.4 gr M17 agar poder (DeMan- Rogosa- Sharpe) was dissolved in 200ml distilled water.

10 tablets of PBS were dissolved in 1 litre of distilled water.

All of these staffs were sterilized in 121°C for 15 minutes. Sterilization procedure took about 2 hours.

Aflatoxin M1 (sigma) was obtained from *Aspergillus flavus* with the concentration of 2/5 µg/ml. AFM1 was diluted with 2ml methanol and was shaken gently.

15 ml of MRS broth was transferred to four sterile universal bottles (60 ml MRS broth). Then yoghurt starter culture (Ascott freeze dried yoghurt culture) which contains *Strptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were added into four universal bottles and shaken gently. These broth cultures were labelled and incubated in 37°C for 24 hours. After 24 hours the growth was observed in the universal bottles.

Solid MRS and M17 agars were put in N₂ stream for 1 hour and transferred to 56°C water bath. Both of molten agars were transferred to 10 sterile petri dishes (5:5). After 15 minutes, 10 nutrient agars plates were streaked with 1 loopful from the bacteria broth cultures. The growth cultures were incubated in 37°C for 24 hours. After 24 hours, most of the growth was observed in M17 agar plates.

Pasteurised milks (whole milk, semi skimmed, skimmed milk) were purchased from Tesco stores. Dr. Onken pasteurized yoghurts (high fat, low fat, fat free) were purchased from Tesco stores. These samples were kept at 4°C in fridge. The milk and yoghurt samples were analysed by Aflascan (Immunoaffinity columns) method. The detection of AFM1 is semi quantitative in this method. The aflascan system provides a rapid, economical semi-quantitative screening test for detection of aflatoxins. This procedure is based on monoclonal antibody bound in an affinity columns format. This method is a very specific and sensitive. This system has capability of detecting total aflatoxin levels to

the required limits of European and International Regulations. Semi- quantitative results were obtained by assessing under the UV light. The fluorescence and intensity of captured AFM1 from the milk or yoghurt samples showed the level of AFM1 in samples.

Aflascan unit is included a pump unit, a glass syringe barrel and an Immunoaffinity column. Aflascan columns should be at fridge temperature. The units must firmly and carefully attach. Also, this method required methanol, chloroform, PBS, UV light box and flurosil tips. R- Biopharm Rhone Limited was the provider of Immunoaffinity columns and flurosil tips.

Milk and yoghurt samples were analysed for any contamination. 10 ml of each milk samples (whole milk, semi skimmed and skimmed milk) were passed through the Immunoaffinity columns and no contamination was detected. Milk samples were added straight to aflascan but yoghurt samples were dissolved in distilled water (1: 15). Yoghurt samples were dissolved in equal, 1 in 4 and 1 in 10 volumes but they did not pass through the columns. Yoghurt samples (high fat, low fat, fat free) have shown no contamination.

RESULTS AND DISCUSSION

Before contaminating the milk samples, the PH of each sample were measured. Whole milk: 6.96, semi skimmed milk: 6.92 and skimmed milk: 6.94.

40µl of AFM1 (100 ng/ml) was added to 20 ml of milk samples (whole milk, skimmed milk, semi skimmed milk) and incubated for about 2 hours in 37°C. Then 10 ml of each milk sample was passed through the column and washed with 20 ml of PBS to remove any residues. 1ml of methanol was passed to denature the antibodies and collected in small test tube. Then 1ml of PBS and 1ml of chloroform were transferred to test tube and shaken gently. Aflatoxins are much soluble in chloroform than in hydrophilic solvents. Therefore, the chloroform was used in the extraction process. The bottom layer of solution was transferred to aflascan and the Immunoaffinity column was removed and flurosil tip was replaced. The solution was passed through flurosil tip. Then the tip was put under the UV light in 360nm wave length to detect the level of toxicity. The intensity of blue light shows the concentration of AFM1. The concentration of AFM1 before and after adding yoghurt bacteria was shown in Table 1 and 2.

After detecting the toxicity level, 400µl of starter culture broth were transferred to each milk samples and incubated for 48 hours in 37°C. After two days, all of the milk samples were converted to yoghurt. The samples were diluted in distilled water (1:15) and passed through the Immunoaffinity columns.

As the level of yoghurt (milk samples) in diluted solution was very low, another extraction method was applied. After adding starter culture broth to contaminated samples and incubating them, 10 ml of three milk samples (whole milk, semi skimmed milk and skimmed milk) were mixed with 10 ml of chloroform and shaken for 2-3 minutes. After 10

minutes the solution separated. The chloroform was collected from each sample to test tubes. The chloroform of samples evaporated overnight. Then samples were re-dissolved in 2ml of methanol and diluted in 8 ml of distilled water. At the end, all samples were passed through the Immunoaffinity columns.

Aflatoxins are not destroyed by heat treatment methods. After each experiment the AFM1 residues must remove from laboratory equipment and stuffs. For this purpose, at the end of each lab sodium hypochlorite and water solution was prepared (2: 10) and all of the reusable and disposable stuffs were soaked in this solution properly overnight. Then, all of them were rinsed. The disposables were put in red bag bins and reusable stuffs were washed and rinsed correctly. The PH of yoghurt samples was measured. High fat yoghurt: 4.32, low fat yoghurt: 4.32 and fat free yoghurt: 4.39.

20 µl of AFM1 (50 ng/ml) was added to 5ml of three yoghurt samples (high fat, low fat and fat free). They all were diluted in 1: 15 and incubated in 37°C for 2 hours. Under the UV light box the level of detection was too low (AFM1<4ppb). According to the results, four assumptions were suggested:

- Higher concentration of AFM1 required per ml of each sample.
- Yoghurt bacteria reacted immediately and bound with AFM1
- The dilution factor was too high and decreased the amount of toxin per ml of sample.
- The extraction method was not appropriate.

The extraction method was changed. Yoghurt samples were contaminated with 20µl of AFM1 in 5ml of each sample. Samples were dissolved in 80% methanol (1ml yoghurt: 4ml methanol). The solution was too thick and 2ml of solution hardly passed through the column. Again the amount of toxin was low and the experiment was not successful.

The other method was based on centrifuge. 20 ml of each sample (high fat, low fat, fat free) were contaminated with 100µl of AFM1 and dissolved in 20ml of 80% methanol. Samples were centrifuged in 3000 for 10 minutes. The upper layers were passed through the columns (positive controls). 10 ml of each sample was analysed by aflscan method and the concentration of AFM1 was detected under the UV light box.

Yoghurt already contains lactic acid bacteria but in some products lactic acid bacteria are removed. For this purpose, yoghurt samples were cultivated in MRS agar and incubated in 37°C for 24 hours. No lactic acid bacteria were detected in the culture media. Therefore, the starter culture was added to contaminated yoghurt samples and incubated in 37°C for 24 hours.

Another suggested method was the same as the milk analysis method. 20 ml of each yoghurt sample (high fat, low fat, fat free) were contaminated with 100µl (250 ng/ml) of AFM1 and incubated in 37°C for 2 hours. Each sample (10ml) was diluted in 10 ml chloroform and mixed. After 10 minutes the solutions separated and the chloroforms were transferred to other test tubes. The chloroform evaporated

overnight and re-dissolved in 2ml of methanol. The solution was diluted in 8ml of distilled water and the concentration of toxins was measured by aflscan method (positive controls).

Next, 400µl of starter culture broth were added to 20ml of contaminated yoghurt samples then incubated in 37°C for 24 hours. The same extraction method was repeated and the level of AFM1 was detected under the UV light box. The Table 1 shows the concentration of AFM1 in whole milk, semi-skimmed milk and skimmed milk before and after using yoghurt starter cultures. The high percentage of AFM1 binding ability by Yoghurt bacteria is observed.

Some losses are observed in concentration of AFM1 before adding yoghurt starter culture in Table 2. It is assumed that during extraction or incubation of contaminated samples some amounts of AFM1 was disappeared.

Column charts were designed for each milk samples (whole milk, semi skimmed milk and skimmed milk) and each milk sample was compared before and after adding yoghurt bacteria in Fig 1, 2 and 3 respectively and concentration of AFM1 were analysed before and after adding yoghurt bacteria (Table 3). Each sample was assessed four times to increase accuracy and decrease probable bias.

The contamination range in whole milk samples are between 30- 40 (ppb) but after adding yoghurt bacteria the toxicity decrease significantly even in one sample no toxicity is observed. In other three samples toxicity are around 4 ppb.

The semi- skimmed milk chart shows the concentration of AFM1 in 4 samples 35, 35, 40 and 35 (ppb) and

Table 1: The concentration of AFM1 before and after adding yoghurt bacteria (50ng/10ml).

Whole milk (ppb)		Semi skimmed milk (ppb)		Skimmed milk (ppb)	
Before	After	Before	After	Before	After
40	4	35	4	35	4
35	4	35	0	30	0
35	4	40	0	35	0
30	0	35	4	35	0

Table 2: Comparison of milk samples.

Milk samples	X±SX
Whole milk	32±1.35
Semi-skimmed milk	34.35±2.14
Skimmed milk	32.75±1.31

Table 3: Concentration of AFM1 before and after adding yoghurt bacteria (125 ng/10 ml).

High fat yoghurt (ppb)		Low fat yoghurt (ppb)		Fat free yoghurt (ppb)	
Before	After	Before	After	Before	After
20	20	20	20	25	20
20	25	25	25	20	20
25	20	25	20	20	25
20	20	25	25	20	20

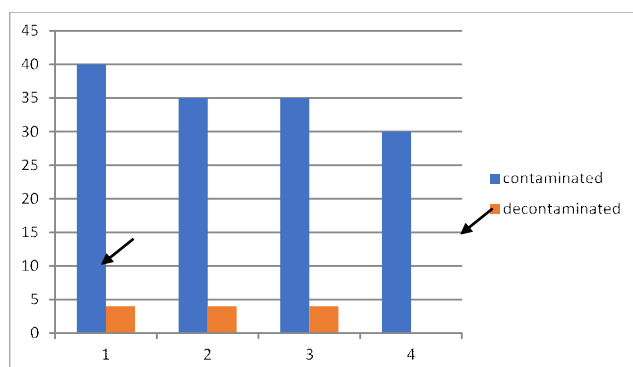


Fig 1: Comparison of whole milk before and after applying yoghurt bacteria.

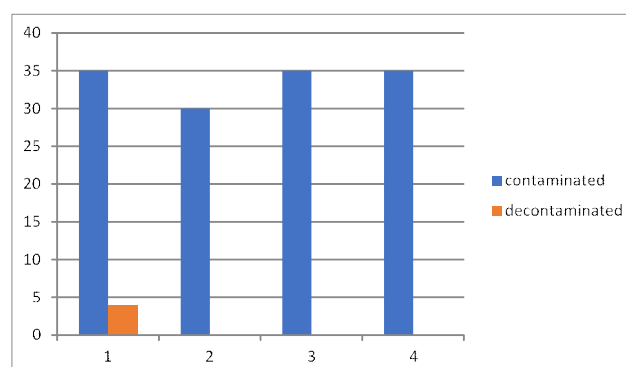


Fig 3: Comparison of skimmed milk before and after applying yoghurt bacteria.

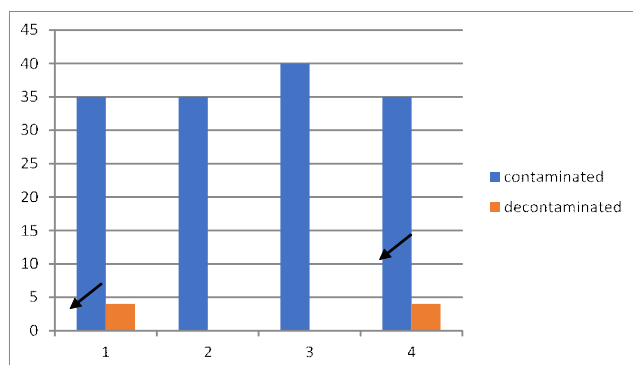


Fig 2: Comparison of semi skimmed milk before and after applying yoghurt bacteria.

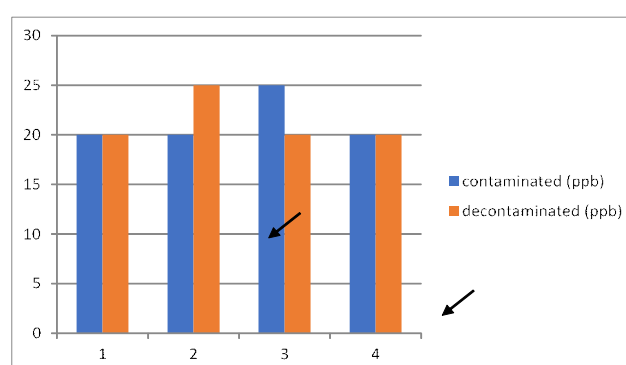


Fig 4: Comparison of high fat yoghurt before and after applying yoghurt bacteria.

decontaminated samples are 4, 0, 0 and 4. The presence of AFM1 is very low and in two samples no toxicity are observed.

Also, skimmed milk samples determine no toxicity in three samples and in one sample the level of AFM1 is very low (4ppb).

The variance analysis (two related samples) was done for determining the difference amount of AFM1 before and after using yoghurt bacteria.

Yoghurt bacteria showed significantly high ($p < 0.05$) percentage of binding ability of AFM1 and there was not notably difference between the milk samples. The fat content of milk samples does not have any effect on AFM1 reduction.

ANOVA variances analysis was also done for comparison of three milk samples (whole, semi skimmed and skimmed). As the Confidence Interval (95%) straddled zero, there was not any significant difference between three samples.

Yoghurt samples results

The Fig 4, 5 and 6 in terms of comparison of high fat, low fat and fat free yoghurt samples represent the concentration of AFM1 in high fat, low fat and fat free yoghurts. The yoghurt bacteria could not be able to degrade the amount of AFM1 and after applying yoghurt starter culture the concentration of AFM1 in yoghurt sample remains stable and no considerable changes was observed.

50 μ l (125 ng) AFM1 was added to 10 ml yoghurt samples but the table above shows the concentration of

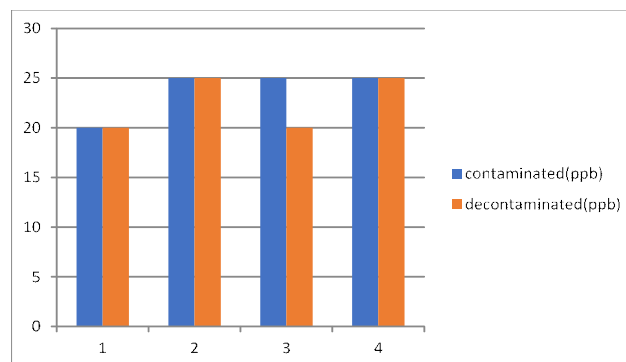


Fig 5: Comparison of low fat yoghurt before and after applying yoghurt bacteria.

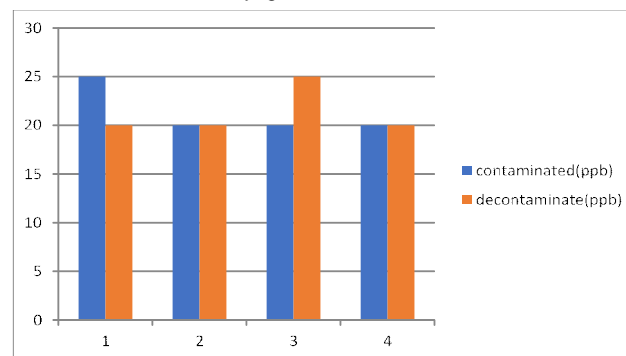


Fig 6: Comparison of fat free yoghurt before and after applying yoghurt bacteria.

AFM1 between 20- 25 (ppb) which is great loss. The concentration of milk was 2/5 times lower than yoghurt (50 ng/ 10 ml) but the levels of toxins under the UV light box were higher than yoghurt samples.

Three charts were designed to compare the levels of AFM1 before and after adding yoghurt bacteria. The charts show no significant decrease in concentration of AFM1 and in some samples the increase in levels of AFM1 are observed.

In 50% of high fat yoghurt samples no changes are observed but in 25% of samples slight decrease is detected. In another 25% an increase is observed.

In low fat yoghurt, the levels of AFM1 remain stable in 75% of samples where as 25% of samples show low decrease.

The concentration of AFM1 in fat free yoghurt samples are 25, 20, 20 and 20. After adding yoghurt bacteria the results are 20, 20, 25 and 20. In two samples no changes are observed but in one sample a slight decrease is detected (20%). Also, one sample shows an increase in amount of toxin (20%).

Also, the fat content of yoghurt samples did not have any effects on concentration of AFM1 because in statistical analysis the confidence interval straddled zero (95%) and no significant change was observed.

CONCLUSION

Several types of aflatoxin (14 or more) occur in nature, but four - aflatoxins B1, B2, G1 and G2 are particularly dangerous to humans and animals as they have been found in all major food crops; but most human exposure comes from contaminated nuts, grains and their derived products. Additionally, aflatoxin M1 (AFM1), a product of aflatoxin B1 (AFB1) metabolism, can be found in milk in areas of high aflatoxin exposure. Subsequently humans may be exposed to this aflatoxin through milk and milk products, including breast milk, especially in areas where the poorest quality grain is used for animal feed. Food crops can become contaminated both before and after harvesting (Peracia *et al.*, 1999). Pre-harvest contamination with aflatoxins is mainly limited to maize, cottonseed, peanuts and tree nuts. Post-harvest contamination can be found in a variety of other crops such as coffee, rice and spices. Improper storage under conditions that favor mould growth (warm and humid storage environments) can typically lead to levels of contamination much higher than those found in the field (WHO report, 2017). In conclusion, the results in this experiments show that the yoghurt bacteria (*S. thermophilus* and *L. Delbrueckii* subsp. *Bulgaricus*) are able to reduce the levels of AFM1 in milk during the fermentation process. On the other hand, yoghurt bacteria were not able to decrease the concentration of AFM1 in contaminated yoghurt samples. Also, the fat content of milk and yoghurt samples did not have any positive or negative effects on AFM1

biodegradation. It could be advised to use yoghurt bacteria for detoxification of AFM1 from foods and feeds. More research is needed to assess the various strains of harmless bacteria, new methods and different conditions to degrade the levels of mycotoxins in foods and feeds and overcome to this worldwide concern.

REFERENCES

- Celik, T.H., Sarimehmetoglu, B., Kupulu, O. (2005). Aflatoxin M1 contamination in pasteurised milk. *Veterinarski Arhiv*. 75(1): 57-65.
- Christou, P., Miller, K.D., Paau, A. (1985). Chemical conversion of aflatoxin B1 to M1. *Phytochemistry*. 24(5): 933-935.
- Egmond, H.P. (1983). Mycotoxins in dairy products. *Food Chemistry*. 11(6): 289-307.
- El-Nezami, H., Kankaanpaa, P., Salminen, S., Ahokas, J. (1998). Ability of dairy strains of lactic acid bacteria to bind a common food carcinogen, Aflatoxinb1. *Food and Chemical Toxicology*. 36(11): 321-326.
- Evaluation of certain contaminants in food (Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series WHO Technical Report Series, No.1002, 2017.
- Fallah, A.A., Jafari, T., Fallah, A., Rahnama, M. (2009). Determination of aflatoxin M1 levels in Iranian white and cream cheese. *Food and Chemical Toxicology*. 47(1): 1872-1875.
- Govaris, A., Roussi, V., Koidis, P.A., Botsoglou, N.A. (2002). Distribution and stability of aflatoxin M1 during milk production and storage of yoghurt. *Food Additives and Contaminants*. 19(11): 1043-1050.
- Haskard, C.A., El-Nezami, H.S., Kankaanpaa, P.E., Salminen, S., Ahokas, J.T. (2001). Surface binding of aflatoxin B1 by lactic acid bacteria. *Applied and Environmental Microbiology*. 67(7): 3086-3091.
- Hussein, H.S., Brasel, M.J. (2001). Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology*. 167(7): 101-134.
- Huwig, A., Freimund, S., Kappeli, O., Dutler, H. (2001). Mycotoxin detoxification of animal feed by different adsorbents. *Toxicology Letters*. 122(9): 179-188.
- Laciaková, A., Cicoová, P., Máté, D., Laciak, V. (2008). Aflatoxins and possibilities for their biological detoxification. *Medycyna Wet*. 64(3): 276-279.
- Manetta, A., Di Giuseppe, L., Giammarco, M. (2005). High-performance liquid chromatography with post-column derivatisation and fluorescence detection for sensitive determination of aflatoxin M1 in milk and cheese. *Journal of Chromatography A*. 1083(5): 219-222.
- Peracia, M., Radic, B., Lucic, A., Pavolovic, M. (1999). Toxic effects of mycotoxins in humans. *Bulletin of the World Health Organization*. 77(9): 754-764.
- Philips, T.D. (1999). Dietary clay in the chemoprevention of aflatoxin-induced disease. *Toxicological Sciences*. 52(1): 118-126.
- Rustom, Y.S. (1997). Aflatoxin in food and feed: Occurrence, legislation and inactivation by physical methods. *Food Chemistry*. 59(1): 57-67.