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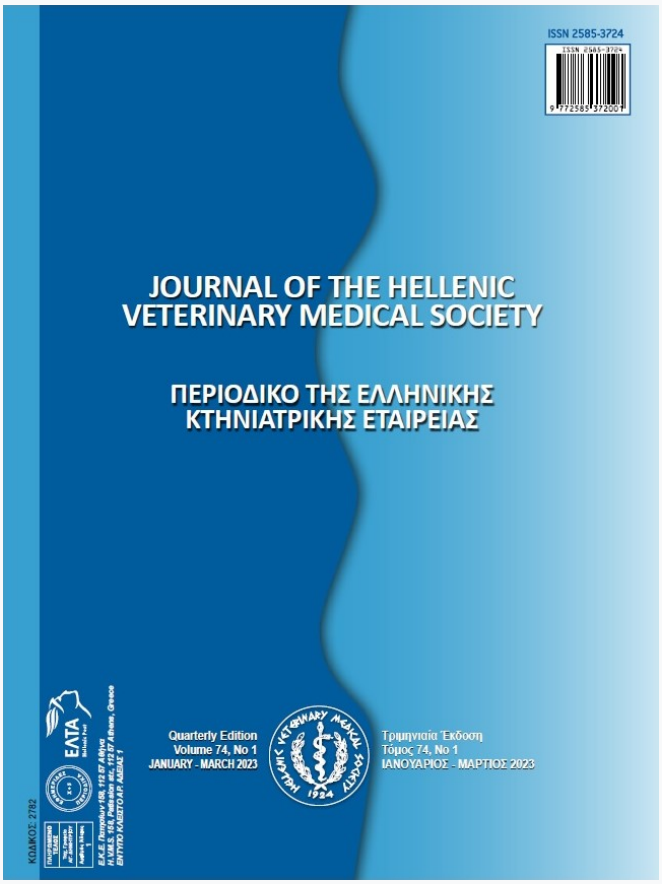

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Research article
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Determination of Aflatoxin M1 levels in Turkish cheeses provided from different regions of Turkey

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ABSTRACT: One of the factors that can affect the hygienic quality of milk and dairy products is aflatoxins. Aflatoxins are produced by moulds, and it is known that the Aflatoxin M1 type, which can be found in milk and dairy products, is a potential risk for public health. In this study, the presence of Aflatoxin M1 in white and tulum cheese samples collected from three different provinces of Turkey (Burdur, Bursa, and Elazig) was investigated with the ELISA method. 42 white and 42 tulum cheese samples (total 84) were analyzed, and the samples were evaluated in terms of their compliance with the limits of the Turkish Food Codex. According to the results; 34 (40.47 %) of 84 cheese samples were contaminated with Aflatoxin M1, ranging from 250 to 559 ng/kg. Fifty (59.52 %) cheese samples were found below the detection limit, meaning they are negative in terms of Aflatoxin M1 and do not pose a risk. On the other hand, 2 samples (2.38 %) were over the tolerance limit of the Turkish Food Codex. Also, the incidence of Aflatoxin M1 in white cheeses was 35.71 % and 45.23 % in tulum cheeses. Although it is not much above the legal limit, Aflatoxin M1 contamination in cheese may reduce the food quality and adversely affect human health. Therefore, it should be treated more carefully in the production of milk and dairy products. In addition, quality feeds should be used in the feeding of dairy animals.

Keywords: aflatoxin M1; white cheese; tulum cheese; public health; ELISA

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INTRODUCTION

Milk is an important food source for human health thanks to its calcium, phosphorus, protein, and other nutrients.It is recommended that milk and dairy products should be consumed in both childhood and adulthood for adequate and balanced nutrition (Iqbal et al., 2015; Miller et al., 2000; Acaroz et al., 2020). The hygienic quality of raw milk and products made from milk is among the most fundamental issues of food hygiene.Many microorganisms that can be found in raw milk can be destroyed by heat treatments such as pasteurization and sterilization (Mungai et al., 2015; Kucuk and Yibar, 2019). Aflatoxins,which can be found in raw milk and dairy products, are resistant to heat treatments and consumption of aflatoxin-containing foods causes serious health problems (Bakirci, 2001; Park, 2002).

Aflatoxins are one of the mycotoxins with high toxicity. Mycotoxins are produced by some moulds sush as *Aspergillus, Penicillium, Fusarium spp* (Kumar et al., 2008). The most common of these is *Aspergillus spp*. They growunder particular temperature and humidity conditions and produce aflatoxin. *Aspergillus flavus, Aspergillus parasiticus, Aspergillus nomius* are some of the important species of this mould (Cheraghali et al., 2007; Zinedine and Manes, 2009). Aflatoxins are divided into 4 different groups: Aflatoxin B1 (AFB1), Aflatoxin B2, Aflatoxin G1, and Aflatoxin G2. *Aspergillus flavus* produces B aflatoxins (B1, B2). AFB1 isvery toxic and carcinogenic in mammals (Hussain and Brasel, 2001; Creepy, 2002). It can be found in nuts, dried fruits, spices, feeds, wheat, corn, and other grains (Colak et al., 2006; Yentur et al., 2006). Aflatoxin M1 (AFM1) which can be found in milk and dairy products, is the metabolic product of AFB1. When the lactating animals consume aflatoxin-contaminated feed, AFB1 is metabolized in the animal body and converted into AFM1 (Fallah et al., 2009; Kav et al., 2011). AFM1 can be detected in milk within 12-24 hours (Sibanda et al., 1999). Since this toxin cannot be removed by heat treatment, it is likely to be found in dairy products such as cheese, yoghurt, butter, and ice cream (Karadal et al., 2018). Besides its carcinogenic, mutagenic, teratogenic effects, the consumption of aflatoxin-contaminated foods can cause suppression of the immune system in humans. In some studies, it is reported that AFM1 is detected in human breast milk (Ozdemir and Kuyucuoglu, 2007; Dinleyici et al., 2018).

Due to its toxic effects, many countries have introduced legal regulations on aflatoxin to protect public health. According to the European Union Commission, the maximum AFM1 level in milk and dairy products is 50 ng/kg (EC, 2010). In the Turkish Food Codex Contaminants Regulation, the maximum limit of AFM1 in milk is specified as 0.05 μg/kg = 50 ng/kg and 500 ng/kg for cheese (TFC, 2008).

With different types and flavors, more than 130 cheese species are produced in Turkey. White cheese, kashar cheese, tulum cheese are the most produced and consumed of them. White cheese is a brined cheese type with salty, acid taste and can be soft or semihard texture. It usually has a maturity period of 3 months (Dagdemir et al., 2003). Tulum cheese is a semi-hard cheese produced usingtraditional methods and is ripened in goatskin bags or plastic materials (Hampikyan et al., 2010; Erkan et al., 2018).Different animal milks can be used in the production of tulum cheese (cow, ewe, or goat) and the maturation process of it is long (Hayaoglu et al., 2007).

The aim of this study was to determine the presence of AFM1 contamination in white and tulum cheese samples in different regions of Turkey.

MATERIALS AND METHODS

Materials

In this study, a total of 84 cheese samples (42 white cheeses and 42 tulum cheeses) were collected from Burdur, Bursa, and Elazig, three different provinces of Turkey. The number of samples taken from each province was equal to each other. Samples were obtained from different producers in public markets and bazaars between November and December 2020. They were brought to the laboratory with cold chain application and stored at 2-4 °C during the process. While bovine milk was used in the production of the collected white cheese samples; at least one of the cow, sheep, goat milk was used in the production of tulum cheeses. All cheese samples used in the study have a maturation period of at least 3 months.

Methods

The Enzyme Linked Immunosorbent Assay (ELISA) method was used to determine AFM1 concentration in cheese samples.The sensitivity of ELISA method is high and it is the most commonly used method in AFM1 analysis.

Test kit principle

The AFM1 extraction and ELISA test procedure

were performed according to the Elabscience test kit manual (Elabscience Biotechnology Inc., USA, Catalog No: E-TO-E018 96T). This kit uses Competitive-ELISA as the method. It can detect AFM1 in samples, such as milk, milk powder, and yoghurt. The kit is composed of ELISA Microtiter plate, Horseradish Peroxidase conjugate, antibody working solution, standard, and other supplementary reagents. The microtiter plate provided in this kit has been pre-coated with coupled antigen. During the reaction, AFM1 in the samples or standart competes with coupled antigen on the solid phase supporter for sites of anti-AFM1 antibody. Then HRP conjugate is added to each microtiter plate well, and substrate reagent is for color development. There is a negative correlation between the optical density value of samples and the concentration of AFM1. The concentration of AFM1 in the samples can be calculated by comparing the optical density of the samples to the standard curve. The calibration curve used in the quantitative evaluation is given in Figure 1.

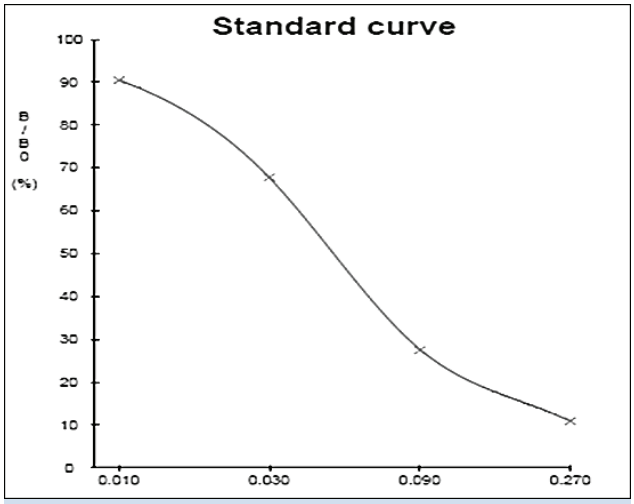

Figure 1. AFM1 analysis calibration curve

Pretreatment of cheese samples

1 gram of cheese sample was weighed and 1 ml of methanol was added. It was oscillated for 1 minute and mixed fully. The mixture was centrifuged at 4000 r/minute for 5 minutes at room temperature. Later, 80 µL of supernatant was taken into another tube and 920 µL of sample diluent was added. After mixing, 50 µL of this extract was used for analysis.

Application of ELISA test

Fifty µL of standard solutions and samples were added to the wells. After adding 50 µL of antibody working solution to each well, the plate was covered with sealer. It was oscillated for 10 seconds to mix thoroughly and then incubated at 25 °C for 60 minutes under shading light. Next, the liquid of each well was removed. 260 µL of wash buffer solution was added and the washing procedure was repeated four times at 30 seconds intervals. Later, 100 µL of HRP (Horseradish Peroxidase) conjugate was added to each well and incubated at 25 °C for 30 minutes. The wells were washed again. Then, 50 µL of substrate reagent A and B was dispensed to the wells and incubated for 15 minutes. The reaction was stopped by 50 µL of stop solution, and the absorbance was measured at 450 nm with microplate reader. The percentage absorbance of the samples was calculated as described by the test kit manual: % absorbance = (average absorbance of standard solution or sample/average absorbance of 0 ppb standard solution) x 100. The obtained values were multipled by the dilution factor of 25. The detection limit of the kit was 250 ng/kg. The obtained data were analyzed in Excel 2019, and the results were expressed as mean±SD (standard deviation).

RESULTS

The results obtained in the study are presented in the tables below. As seen in Table 1; 34 cheese samples (40.47%) were contaminated with AFM1 in total. The percentages of contamination in white and tulum cheeses were 35.71% and 45.23%, respectively. The number of samples below the detection limit (250 ng/kg) was determined as 50 (59.52%). It is seen that the number of negative samples is higher than the number of positive samples. Also, the mean concentrations of the positive white and tulum cheese samples were 336.86±57.61 and 372.52±94.90, respectively.

Table 1. Aflatoxin M1 levels of cheese samples

Samples (n)	Number of samples n (%)		Concentration (ng/kg)		Positive samples (ng/kg)
	Positive	Negative	Minimum	Maximum	Mean±SD
White cheese (42)	15 (35.71%)	27 (64.28%)	268	478	336.86±57.61
Tulum cheese (42)	19 (45.23%)	23 (54.76%)	251	559	372.52±94.90
Total (84)	34 (40.47%)	50 (59.52%)	251	559	356.79±81.51

Table 2. Contamination levels and ranges of AFM1 in cheese samples n (%)

Cheese type	<250 ng/kg	250-300 ng/kg	300-350 ng/kg	350-400 ng/kg	400-500 ng/kg	>500 ng/kg
White cheese (42)	27 (64.28)	6 (14.28)	6 (14.28)	1 (2.38)	2 (4.76)	-
Tulum cheese(42)	23 (54.76)	4 (9.52)	5 (11.90)	3 (7.14)	5 (11.90)	2 (4.74)
Total (84)	50 (59.52)	10 (11.90)	11 (13.09)	4 (4.76)	7 (8.33)	2 (2.38)

Table 3. Distribution of Aflatoxin M1 levels by provinces

	White cheese n (%)			Tulum cheese n (%)		
Province	Number of samples	Positive	Negative	Number of samples	Positive	Negative
Burdur	14	6 (42.85)	8 (57.14)	14	6 (42.85)	8 (57.14)
Bursa	14	6 (42.85)	8 (57.14)	14	5 (35.71)	9 (64.28)
Elazig	14	3 (21.42)	11 (78.57)	14	8 (57.14)	6 (42.85)

In Table 2, AFM1contamination levels and ranges of cheese samples were given.Based on these results, there were 2 samples exceeding the maximum limit of the Turkish Food Codex (2.38%). AFM1 distribution in cheese samples was evaluated in Table 3 by Burdur, Bursa, and Elazig provinces. In white cheese,while there were equal numbers of positive samples in those collected from Burdur and Bursa provinces (6; 42.85%), the least positive samples were found in those from Elazig (3; 21.42%). In terms of tulum cheese, the sample from Bursa had the least positive samples (5; 35.71%) while the sample from Elazig had the most positive samples with 8 (57.14%).

DISCUSSION

Aflatoxin in dairy products is an important public health problem worldwide.Many studies have been carried out in Turkey and also other countries for the determination of AFM1 in milk and dairy products, which has strong toxigenic properties (Table 4). In these studies, various cheese types were used (kashar, tulum, white, ewe, cube, feta-type, cream, etc.).

In the present study, 35.71% of white cheese and 45.23% of tulum cheese were found to be positive (40.47% in total). Hampikyan et al. (2010) reported that 60% of white cheese and 55% of tulum cheese samples, obtained from Istanbul, were contaminated with AFM1 in the range of 0.052-2.52 μg/kg. They also stated that 13.3% and 10% of these samples were above the legal limit. In our study, while there were no samples exceeding the limit in white cheeses, 4.74% of tulum cheese samples were found to exceed the legal limit. In a study performed with Turkish cheeses, 30 of the 304 samples (9.9%) were found above this limit (Aydemir Atasever et al., 2010). Kure and Skaar (2019) reported that while fungi can grow on all cheese types, due to the high water activity fungal contamination happens more easily on soft cheeses. In terms of higher aflatoxin levels in tulum cheeses, the results of our study do not agree with this.

In another study conducted in Elazig, AFM1 was detected in all tulum cheese samples (Erkan et al., 2018).According to the studies using different types of cheese in Turkey; 68.8% of kashar cheese, 6.25% of Urfa cheese, and 1.1% of cube cheese samples were found to be positive by AFM1 (Gunsen and Buyukyoruk, 2003; Ardic et al., 2008; Agaoglu et al., 2020).

It has been revealed that AFM1 is cytotoxic in human liver cells under in vitro conditions. In addition, it can cause DNA damage, gene mutations, and chromosomal abnormalities in mammals. In order to prevent these possible risks, the World Health Organization recommends minimizing Aflatoxin M1 levels in milk and dairy products (Mehenktas, 2019).

When studies conducted in different countries were examined, it was found that cheese samples were contaminated with aflatoxin at low or high levels. Montagna et al. (2008) analyzed 265 cheese samples produced from different milks in Italy. In 44 of these samples (16.6%) AFM1 was detected in concentrations between 50 and 250 ng/kg. Elkak et al. (2012) reported that AFM1 was found in 67.56% of the local cheese samples in Lebanon and 17.33% of the samples were above the 250 ng/kg.In a study, analyzing white cheese samples in Iran, the incidence of AFM1 was 60%, with a range of 40.9 ng/kg to 374 ng/kg (Tavakoli et al., 2012). Dasthi et al. (2009) analyzed 40 cheese samples in Kuwait and found that 32 of them (80%) were contaminated with AFM1. They also reported that one sample was above the 250 ng/kg.

Table 4. Incidence of AFM1 in different cheese types and locations

n	n_1 (%)	Cheese type	AFM1 levels (min-max)	Location	References
183	121 (65)	White cheese	40-4890 ng/kg	Istanbul, Turkey	Aycicek et al., 2002
125	86 (68.8)	Kashar cheese	10-740 ng/kg	Bursa, Turkey	Gunsen and Buyukyoruk, 2003
600	30 (5)	White, kashar, and processed cheese	100-800 ng/kg	Bursa, Turkey	Yaroglu et al., 2005
39	11 (28.21)	White, feta-type, and tulum cheese	<50-188.44 ng/kg	Ankara, Turkey	Gurbay et al., 2006
64	4 (6.25)	Urfa cheese	51.10-99.60 ng/kg	Sanliurfa, Turkey	Ardic et al., 2008
80	41 (51.3)	White, kashar, and tulum cheese	0.052-2.52 µg/kg	Istanbul, Turkey	Hampikyan et al., 2010
45	40 (88.9)	White cheese	55-600 ng/kg	Burdur, Turkey	Kocasari et al., 2012
100	100 (100)	Tulum cheese	0.64-4.32 µg/kg	Elazig, Turkey	Erkan et al., 2018
80	51 (63.75)	White and tulum cheese	25.30-201.27 ng/kg	Afyonkarahisar, Turkey	Acaroz, 2019
90	1 (1.1)	Cube cheese	2.16-53.94 ng/kg	Sivas, Turkey	Agaoglu et al., 2020
20	15 (75)	White cheese	0.11-0.52 ng/g	Libya	Elgerbi et al., 2004
265	44 (16.6)	Sheep, cow, buffalo, goat cheese	50-250 ng/kg	Italy	Montagna et al., 2008
40	32 (80)	Cheese	23.8-452 ng/kg	Kuwait	Dashti et al., 2009
210	161 (76.6)	White and cream cheese	52.1-785.4 ng/kg	Iran	Fallah et al., 2009
48	13 (27.1)	Minas Frescal, Minas Padrão cheese	0.030-0.313 ng/g	Brazil	Oliveira et al., 2011
111	75 (67.56)	Halloumi, Naboulsi, Feta, Baladi, Akkawi cheese types	1.26-315 ng/kg	Lebanon	Elkak et al., 2012
50	30 (60)	White cheese	40.9-374 ng/kg	Iran	Tavakoli et al., 2012
40	15 (53.85)	White and soft cheese	75.35-300.7 ng/L	Baghdad	Al Mossawei et al., 2016
30	13 (43.33)	Domiati and processed cheese	12.50-74.23 ng/kg	Egypt	Tahoun et al., 2017
46	39 (85)	Cheese	2.53-217.15 ng/kg	Qatar	Hassan et al., 2018

n: Number of samples
n_1: Positive samples

Many factors are effective in causing aflatoxin in milk and dairy products, especially geographical differences and seasons.The cheese type and production technique vary between regions and countries.In addition, the hygienic quality of the milk used in production, the production time, and the nutrition of the dairy animals are other important factors (Battacone et al., 2005; Iha et al., 2011). Seasonally, more AFM1 can be seen in winter milk because animals cannot be fed with green and fresh grass in winter but are mostly fed with grain-based feed (Kamkar et al., 2014; Aksoy and Sezer, 2019).Iqbal et al. (2013) examined the AFM1 levels of milk and dairy products collected in summer and winter months by HPLC. While the AFM1 contamination level was found to be 45% in the samples collected in the winter months, this rate was 32% in the summer months.

CONCLUSIONS

In this study, AFM1 contamination in Turkish white and tulum cheeses was investigated, and the results were evaluated. While 40.47% of the samples were positive in terms of AFM1, there were only two samples (2.38%) that did not comply with the legal limits. This toxin is an important public health problem that should not be ignored. It is resistant to processes such as heat treatment, cooling, freezing, fermentation, and drying (Galvano et al., 1996; Park, 2002).As a result, it cannot be completely removed from the milk and its derivatives.

Cheese is an important dairy product that is included in the diet of almost every person. The danger of aflatoxin in cheese, which has many different types

and is consumed extensively around the world, has always maintained its importance.The high contamination levels seen in most of the studies reviewed, revealed that this toxin should be controlled and preventive measures should be taken.

Then, the first preventive measure should be at the feeding stage of milk-giving animals. Next; the feeds used in animal nutrition should be dried very well to prevent mould growth. The feed should not be stored in hot and humid environments. Also, they should be checked regularly in terms oftheir AFB1 levels. In addition, milk and dairy producers should be informed about this issue, and production should be conducted in more hygienic and healthy conditions.International standard values for aflatoxin levels in feeds and foods should be determined.Reliable results should be obtained by developing analysis methods.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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