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Determination of Aflatoxin M₁ Levels in Turkish White and Kashar Cheeses Made of Experimentally Contaminated Raw Milk

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ABSTRACT

Aflatoxin M₁ (AFM₁) is the hydroxylated metabolite of aflatoxin B₁ and may be found in milk and dairy products such as cheese. This study was aimed to determine the AFM₁ levels in Turkish White and fresh Kashar Cheese which were produced with experimentally contaminated raw milk and to observe the change of AFM₁ distribution in White cheese during ripening. For this purpose, AFM₁ was added in concentrations of 0.25, 0.50 and 1.0 µg/L of milk and then, the cheeses were produced according to their technologies. Whey, boiling water, cheese and brine samples were examined for AFM₁ residues. The quantitative analysis of AFM₁ was performed by ELISA using the AFM₁ test kit. The toxin remained as 42.87% and 34.73% in Turkish White and Kashar cheese samples, respectively. The change of AFM₁ concentration during the White cheese ripening of 0-90 days was recorded as the average of 9.8%.

Key words: aflatoxin M₁, aflatoxins, ELISA, Turkish White cheese, Kashar cheese

INTRODUCTION

Aflatoxins are a group of highly toxic secondary metabolic products of some *Aspergillus* spp. such as *A. flavus* and *A. parasiticus*⁽¹⁾. Aflatoxin B₁ (AFB₁) is transformed into 4-hydroxylated metabolite known as "milk toxin" or aflatoxin M₁ (AFM₁) in mammals by the consumption of AFB₁ contaminated diets⁽²⁾. Of all mycotoxins, AFB₁ is considered to be the most toxic compound. International Agency for Research on Cancer (IARC) included AFB₁ as primary and AFM₁ as secondary groups of carcinogenic compounds⁽³⁾.

AFM₁ appears in milk 12-24 hour after ingestion of the first AFB₁-contaminated ration⁽⁴⁾. Milk has the greatest demonstrated potential for introducing aflatoxin residues from edible animal tissues into the human diet⁽⁵⁾. The consumption of milk and dairy products by human especially by infants and young children increases the risk of exposure to AFM₁⁽⁴⁾. Aflatoxins may be found in cheese originating from three possible sources: a) presence of AFM₁ in milk with which cheese are manufactured, as a consequence of food contaminated with AFB₁ eaten by dairy cattle; b) synthesis of AF B₁, B₂, G₁, and G₂ by fungi which grow on cheese such as *Aspergillus* spp., and c) the use of dried milk with AFM₁, to enrich milk employed in cheese production⁽¹⁾.

Regulatory limits throughout the world are influenced by economic considerations and may vary from

one country to another⁽⁶⁾. The European Commission proposes a maximum permissible level of 0.005 µg/kg AFM₁ in milk and milk products⁽⁷⁾. In our country, legal limits for AFM₁ in milk and cheese are 0.005 µg/L and 0.25 µg/kg respectively⁽⁸⁾.

In Turkey, 40-50 cheese varieties are known but only three of them have economic value: Turkish White cheese (Beyaz Peynir), Kashar cheese and Tulum cheese⁽⁹⁾. Turkish White cheese is the most popular traditional cheese which is produced and consumed in Turkey⁽¹⁰⁾. It represents 60-80% of total cheese production⁽⁹⁾. Kashar cheese, a semi-hard Turkish traditional cheese, is also one of the cheeses consumed most in Turkey⁽¹¹⁾. According to Turkish Standards, Kashar cheese is classified as "fresh Kashar cheese" and "old or matured Kashar cheese" in terms of ripening⁽¹²⁾. In recent years, the manufacture of fresh Kashar cheese has increased in contrast to old Kashar cheese because of the economical reasons⁽¹¹⁾.

When cheese is manufactured from AFM₁-contaminated milk, the toxin can be carried over into both whey and cheese⁽¹⁾. Contradictory data have been published about AFM₁ recovery after cheese preparation. Blanco *et al.* (1988) have reported that the distribution of AFM₁ in cheese can be ascribed to factors such as extraction technique, methodology, type and degree of milk quality and the cheese manufacture process⁽¹³⁾. Although several studies have been undertaken to determine the presence of AFM₁ in milk and dairy products in our country, there is a little information about the distribution of AFM₁ in Turkish White and Kashar cheese which

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are produced from contaminated raw milk.

The aim of this study was to determine the AFM₁ residue levels in Turkish White and Kashar cheese which were produced with experimentally contaminated raw milk and to observe the change of AFM₁ distribution in White cheese during ripening.

MATERIALS AND METHODS

I. Chemicals

AFM₁ standard was obtained from Sigma (Sigma-A 6428, Taufkirchen, Germany). Stock solution of AFM₁ was prepared in a methanol/chloroform mixture (81:19, v/v), 50 mg/mL concentration and is kept frozen at -20°C. When using, it was diluted with methanol/chloroform (1:1, v/v) at proper concentrations⁽¹⁾. All other chemicals were supplied by Merck, Darmstadt, Germany.

II. Milk and Starter Cultures

Cow milk (pH 6.6) was obtained from the Veterinary Medicine Research and Practice Farm of Istanbul University. *Lactococcus lactis* and *L. cremoris* (Visbyvac DIP 10u, cheese-mix 2, Wiesby, Germany) and *Streptococcus thermophilus* E (Wiesby GmbH) were used as the starter culture in the manufacture of Turkish White and Kashar cheeses respectively.

III. Cheese Production

(I) Turkish White Cheese

Thirty liters of milk was divided into three equal parts (A, B and C). The milk showed no detectable levels of AFM₁ (data not shown). The toxin was added to the first group (group A) in a concentration of 0.25 µg/L, to the second group (group B) in a concentration of 0.50 µg/L and to the third group (group C) in a concentration of 1.0 µg/L of milk. After the mixing process, each group of contaminated milk was pasteurized at 77°C for 14 sec. The milk was cooled to 32°C, and the starter culture (2 mL/100 mL) and CaCl₂ (0.02 g/100 mL) were added into the milk. The liquid calf rennet (Intermak, Istanbul, Turkey) was added at a level sufficient to coagulate the milk within 90 min (1 g/10 L). Following coagulation, the coagulum was cut into cubes (2-3 cm sides) and allowed to rest for 10 min. The curds were carefully transferred from the cheese vats into the moulds. After 1 hr of draining (without pressing), pressure was applied at room temperature (~21°C) until the whey drainage stopped (~6 hr).

The cheese block was cut into cubes of about 5×5×5 cm³ with a knife. The pieces of curd were placed in the brine (14 g NaCl/100 mL water) for about 12 hr (until the pH reached 5.0) at 21°C. After salting, the cheese

cubes were transferred to plastic boxes and the brine was added to cover the surface of cheese cubes. The cheeses ripened at 4±1°C for 90 days⁽¹⁰⁾. Cheeses were manufactured in triplicate for each group in different dates.

(II) Turkish Fresh Kashar Cheese

Thirty liters of milk was divided into three equal parts (D, E and F). The milk showed no detectable levels of AFM₁ (data not shown). The toxin addition was performed with the same amount as in Turkish White Cheese samples. After mixing, all contaminated groups were pasteurized at 77°C for 14 sec and cooled to 32°C. Starter culture (9 mL/kg) and CaCl₂ (2 mg/kg) were added. When the pH of milk was at 6.2-6.3, rennet diluted with pure water was added to all groups. After 50 min, the curd was cut in the shape of 1 cm³ cubes. The cut curd was allowed to settle for 10 min. Cooking was done by increasing the temperature from 34 to 40°C within 30 min. At the same time the cheese curd was agitated. At end of the cooking, whey was drained from each group until the drainage stopped (~6 hr). The cheese curd was fermented until the pH reached 5.2-5.5 (~10-16 hr). The curd was boiled in brine at 72°C for 3 min and then put into cylindrical plastic moulds. All cheeses were cooled at room temperature and the moulds were removed. Then the cheese blocks were kept for 24 hr at room temperature by rotating every two hr⁽¹¹⁾. Cheeses were manufactured in triplicate for each group in different dates.

The analyses for Kashar cheese were performed in milk, whey, boiling water and produced Kashar cheese samples and for White cheese in milk, whey, produced White cheese samples (at 0 and 90th day of storage) and in brine.

(III) Determination of AFM₁ by Enzyme-Immunoassay

The quantitative analysis of AFM₁ in the samples was performed by enzyme-linked immunoassay (ELISA) using the AFM₁ test kit (Ridascreen, R-Biopharm, Darmstadt, Germany). This method is quick, reliable and cost effective for the estimation of AFM₁⁽¹⁴⁾. Lopez *et al.* (2001) reported that the method has been previously validated by the thin layer chromatography according to the Association of Official Analytical Chemists⁽¹⁾.

(IV) Recovery Studies

Recovery studies were performed in order to control the validity of the method. According to the instructions of the Ridascreen kit, the recovery rate in spiked fatty milk (10-80 pg/mL) is 95% with a mean coefficient of variation (CV) of 15%. In cheese the recovery rate is 102% (CV=11%). Our recovery was tested in milk, whey and boiling water of Kashar cheese by spiking 0.01 ppb and in cheese 0.06 ppb AFM₁ respectively just before the test. The extraction and test procedures were done

as follows. All experiments were carried in triplicate and the mean values were used. The mean recoveries were found to be, 92% for milk (CV=15%), 95% for whey (CV=11%), 97% for boiling water of Kashar cheese (CV=12%) and 100% for cheese (CV=12%).

(V) Preparation of Samples

Sample preparation and ELISA test were performed according to the instructions of the test kit⁽¹⁵⁾. Milk sample (4 mL) was chilled and then centrifuged for 10 min at 3500 ×g (Hettich Universal 16R, Tuttlingen, Germany). An aliquot (100 µL per well) of the skimmed milk was used directly in the test. Cheese samples (2 g each) were homogenized (Ultraturax, Janke&Kunkel, Staufen, Germany) and extracted with 40 mL of dichloromethane. The suspension was filtered and then 10 mL of the extract was evaporated at 60°C under nitrogen. The oily residue was redissolved in 0.5 mL of methanol, 0.5 mL of phosphate buffer (Na₂HPO₄: 1.427% w/v, KH₂PO₄: 0.907 w/v, ratio 8:2, pH 7.2), 1 mL of n-heptane and mixed thoroughly. After centrifugation for 15 min at 2700 rpm, and 100 µL of the methanolic phase was brought to a 10 mL methanol, by adding of 400 µL of Ridascreen Buffer 1. An aliquot (100 µL per well) of this solution was used in the test.

Whey, boiling water and brine samples were examined directly without pretreatment.

(VI) Test Procedures

The AFM₁ standards and test samples in duplicate were added to the wells of micro-titer plate precoated with antibodies against AFM₁ and incubated at room tempera-

ture in the dark for 60 min. After the washing step, AFM₁ peroxidase conjugate was added to the wells and incubated for another 60 min at room temperature (20-25°C) in the dark. The unbound conjugate was removed during washing. Subsequently, 50 µL each of substrate (urea peroxide) and chromogen (tetramethyl-benzidine) were added to the wells and incubated for 30 min in the dark. The reaction was stopped by the addition of 100 µL of 1 M H₂SO₄ and the absorbance was measured at 450 nm in a ELISA plate reader (ELX 800, Bio-tek Inst., Winooski, VT, USA)

(VII) Evaluation and Statistics

The mean values of the absorbances for the standards and the samples were evaluated according to the R1-DAVIN.EXE computer program from R-Biopharm. The mean lower detection limit was 5 ng/L for milk, whey, boiling water, brine and 50 ng/kg for cheese. Standard error for the mean values was calculated by Statistical Package for Social Sciences (SPSS) (version 10.0) for MS/Windows.

RESULTS AND DISCUSSION

In this study, the experimental cheese production was done in June and July. The milk used in production of all kinds of cheese groups showed no detectable levels of AFM₁. In terms of seasonal distribution, the highest number of AFM₁ positive milk samples was reported in spring and autumn⁽²⁾. Applebaum *et al.* (1982) stated that there was no AFM₁ in milk samples which were obtained in summer season⁽¹⁶⁾. In our country, it was reported by Bakirci (2001) that, the highest AFM₁ residue value was

Table 1. The mean AFM₁ levels of three different production groups of White Cheese^a samples

Production group	AFM ₁ in milk (µg/L)	AFM ₁ in cheese (µg/kg)	AFM ₁ in whey (µg/L)	% AFM ₁ in cheese	% AFM ₁ in whey	Total AFM ₁ (µg/kg)	Recovered AFM ₁ (%)
A	0.250	0.102	0.138	42.50	57.50	0.240	96.00
A ^b	0.250	0.101	0.142	41.56	58.44	0.243	97.20
A ^c	0.250	0.104	0.138	42.97	57.03	0.242	96.80
Mean ± SE ^d	0.250 ± 0.00	0.102 ± 0.00	0.139 ± 0.00	42.34 ± 0.42	57.66 ± 0.42	0.242 ± 0.00	96.67 ± 0.35
B	0.500	0.201	0.283	41.53	58.47	0.484	96.80
B ^b	0.500	0.208	0.279	42.71	57.29	0.487	97.40
B ^c	0.500	0.211	0.247	43.50	56.50	0.485	97.00
Mean ± SE	0.500 ± 0.00	0.207 ± 0.00	0.270 ± 0.01	42.58 ± 0.57	57.42 ± 0.57	0.485 ± 0.00	97.07 ± 0.18
C	1.00	0.429	0.546	44.00	56.00	0.975	97.50
C ^b	1.00	0.430	0.546	44.06	55.94	0.976	97.60
C ^c	1.00	0.418	0.553	43.05	56.95	0.971	97.10
Mean ± SE	1.00 ± 0.00	0.426 ± 0.00	0.548 ± 0.00	43.70 ± 0.33	56.30 ± 0.33	0.974 ± 0.00	97.40 ± 0.15
Total average ± SE	0.583 ± 0.11	0.245 ± 0.05	0.319 ± 0.06	42.87 ± 0.31	57.13 ± 0.31	0.567 ± 0.11	97.04 ± 0.16

^aThe White cheese and whey output for 1-L milk are 18-20% and 50-55% respectively.

^bSecond production group.

^cThird production group.

^dStandard error.

found in milk samples collected in April⁽¹⁷⁾.

The mean losses in AFM₁ levels during White cheese production were 57.13% in average for White cheese samples, as compared with cheese milk (Table 1). In Kashar cheese, the losses of AFM₁ levels were detected as an average of 65.27% (in whey 55.40%, in boiling water 9.87%) (Table 3). These results are similar to that by Lopez *et al.* (2001) in Argentina who reported a reduction of about 60% in AFM₁ level in cheese samples during manufacture⁽¹⁾. According to previous studies, distinct results have been reported on the AFM₁ recovery after cheese preparation. Purchase *et al.* (1972) and McKiney *et al.* (1973) found losses of AFM₁ during cheese production of 65% and 47%^(18,19). Beside this, subsequent studies by several researchers reported increases in AFM₁ level in cheese^(16,20). The increase in AFM₁ concentration in cheese has been explained by the affinity of AFM₁ for casein⁽²⁾. Chemically, AFM₁ is a water-soluble compound, and casein molecule has hydrophobic domains so that AFM₁ has affinity to casein of milk⁽¹⁷⁾.

The change of AFM₁ amount during the cheese ripening of 0-90 days can be seen in Table 2. A little reduction of AFM₁ during the White cheese ripening period was recorded as an average of 0.024 µg/kg. Ciegler *et al.* (1966) reported that the microorganisms such as bacteria, moulds, yeasts, actinomycetes and algae in foods and feeds may cause a little decrease in the amount of AFM₁⁽²¹⁾. According to Kaniou-Grigoriadou *et al.* (2005), AFM₁ residue was not detected in the traditional feta cheese although 84.1 ng/kg of AFM₁ was detected in the curds of the same feta cheeses⁽¹⁴⁾. Marshaly *et al.* (1986) reported a gradual decrease in stored Karish cheese⁽²²⁾.

On the other hand, according to a review concerning the AFM₁ incidence in milk and milk products during ripening and storage periods, several kinds of cheeses such as Brick, Camembert, Cheddar, Manchego, Mozzarella, Parmesan, Limburger, Tilsit and Gouda showed an overall stability of AFM₁ levels⁽²⁾.

Presence of AFM₁ in milk and dairy products is a hazard to human health because aflatoxins are carcinogenic, teratogenic and mutagenic. Therefore, studies were undertaken in different countries and also in our country to examine the presence and levels of AFM₁ in milk and dairy products. Several surveys were performed in order to determine the presence of AFM₁ in cheeses which were produced in our country. Demirer *et al.* (1989), Kivanc (1990) and Ozkalp (1992) reported that AFM₁ was not found in cheese samples⁽²³⁻²⁵⁾. Oruc and Sonal (2001) examined AFM₁ levels in milk and cheese from Bursa, Turkey and found AFM₁ in 89.5% of 57 cheese samples with ranges of 0-180 ng/kg. Seven of the samples were found contaminated over maximum permissible level of AFM₁ in Turkish Food Codex⁽²⁶⁾. Gunsen and Buyukyoruk (2002) analyzed 130 cheese samples in Bursa and determined an average of 0.142 µg/kg AFM₁. The amounts of AFM₁ residue in 15% of the samples were found over maximum permissible level of 0.25 µg/kg given in Turkish Food Codex⁽²⁷⁾. Sarimehmetoglu *et al.* (2004) detected AFM₁ contamination in 327 (81.75%) of 400 cheese samples. The numbers of cheese samples that contained AFM₁ over the legal limits of 0.25 µg/kg were 110 (27.5%)⁽²⁸⁾. Gurses *et al.* (2004) analyzed 63 cheese samples in Erzurum for the occurrence of AFM₁. In 28 (44.44%) of 63 samples, AFM₁ was detected in concentra-

Table 2. The mean AFM₁ levels of White Cheese samples during the ripening period

Production group	AFM ₁ in cheese 0 day (µg/kg)	AFM ₁ in cheese 90th day (µg/kg)	Brine 90th day (µg/L)	Reduction amount of 0-90th day (µg/kg)	Reduction amount of 0-90th day (%)
A	0.102	0.092	ND ^d	0.010	9.8
A ^a	0.101	0.090	ND	0.011	10.9
A ^b	0.104	0.091	ND	0.013	12.5
Mean ± SE ^c	0.102 ± 0.00	0.091 ± 0.00	—	0.011 ± 0.00	11.06 ± 0.70
B	0.201	0.180	ND	0.021	10.4
B ^a	0.208	0.191	ND	0.017	8.2
B ^b	0.211	0.192	ND	0.019	9.0
Mean ± SE	0.207 ± 0.00	0.188 ± 0.00	—	0.019 ± 0.01	9.20 ± 0.58
C	0.429	0.395	ND	0.034	7.9
C ^a	0.430	0.389	ND	0.041	9.5
C ^b	0.418	0.374	ND	0.044	10.5
Mean ± SE	0.426 ± 0.01	0.386 ± 0.01	—	0.039 ± 0.03	9.30 ± 0.92
Total average ± SE	0.245 ± 0.14	0.221 ± 0.12	—	0.024 ± 0.01	9.85 ± 0.08

^aSecond production group.

^bThird production group.

^cStandard error.

^dND: AFM₁ was not detected (detection limit: 0.005 µg/L).

Table 3. The mean AFM₁ levels of three different production groups of Kashar Cheese^a samples

Production group	AFM ₁ in milk (µg/L)	AFM ₁ in cheese (µg/kg)	AFM ₁ in whey (µg/L)	AFM ₁ in boiling water (µg/L)	% AFM ₁ in cheese	% AFM ₁ in whey	% AFM ₁ in boiling water	Total AFM ₁ (µg/kg)	Recovered AFM ₁ (%)
D	0.250	0.085	0.136	0.021	35.12	56.20	8.68	0.242	96.8
Db	0.250	0.085	0.134	0.020	35.56	56.07	8.37	0.239	95.6
Dc	0.250	0.086	0.134	0.020	35.83	55.84	8.33	0.240	96.0
Mean ± SE ^d	0.250 ± 0.00	0.085 ± 0.00	0.135 ± 0.00	0.020 ± 0.00	35.50 ± 0.217	56.04 ± 0.11	8.46 ± 0.11	0.240 ± 0.00	96.13 ± 0.35
E	0.500	0.169	0.266	0.045	35.21	55.42	9.37	0.480	96.0
E ^b	0.500	0.163	0.272	0.051	33.54	55.97	10.49	0.486	97.2
E ^c	0.500	0.174	0.260	0.054	35.66	53.28	11.06	0.488	97.6
Mean ± SE	0.500 ± 0.00	0.169 ± 0.00	0.266 ± 0.00	0.050 ± 0.00	34.80 ± 0.65	54.89 ± 0.82	10.31 ± 0.50	0.485 ± 0.00	96.93 ± 0.48
F	1.00	0.330	0.539	0.101	34.02	55.57	10.41	0.970	97.0
F ^b	1.00	0.338	0.530	0.110	34.56	54.19	11.25	0.978	97.8
F ^c	1.00	0.321	0.545	0.106	33.03	56.07	10.90	0.972	97.2
Mean ± SE	1.00 ± 0.00	0.330 ± 0.00	0.538 ± 0.00	0.106 ± 0.00	33.87 ± 0.45	55.28 ± 0.56	10.85 ± 0.24	0.973 ± 0.00	97.33 ± 0.24
Total Average ± SE	0.583 ± 0.11	0.194 ± 0.04	0.313 ± 0.06	0.059 ± 0.01	34.73 ± 0.33	55.40 ± 0.33	9.87 ± 0.40	0.566 ± 0.11	96.8 ± 0.26

^aKashar cheese and whey output for 1-L milk are 10-12% and 60-65% respectively.^bSecond production group.^cThird production group.^dStandard error.

tions between 7 ng/kg and 202 ng/kg⁽²⁹⁾.

In conclusion, cheeses may contain AFM₁ at different levels, because of the presence of AFM₁ in milk. According to our results, during the cheese production, the mean losses of AFM₁ were detected as 57.13% and 65.27% in Turkish White and Kashar cheese respectively, while 42.87% and 34.73% of the toxin remained in cheeses stated above. Regardless of different research results on the ratio of AFM₁ distribution in cheese consensus of opinion is to accept that AFM₁ residues in cheese are hazardous for public health. Because of this, it is important to minimize the mould contamination of the feeds, to prevent animals eating the contaminated feeds and to analyze the feeds periodically for AFB₁ level. Also, milk and milk products have to be analyzed for the AFM₁ residue in order to prevent consuming the ones that carry AFM₁ over officially permissible limits.

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