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RESEARCH ARTICLE

Determination of AFM$_1$ Levels of Mare's Milk and Koumiss Produced in the Highlands of the Kyrgyz Republic

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Abstract

Mare's milk and koumiss are two very important basic foodstuffs in central Asia.This study aimed to research the AFM1 contamination in mare's milk and koumiss samples collected from local producers in the highlands in Naryn, Issyk-Kul, Bishkek and Talas regions of Kyrgyz Republic. For this purpose, 75 raw mare's milk and 75 koumiss samples collected in May, June and July 2017 were analyzed using the ELISA method. AFM1 was detected in 13.3% of the 75 raw mare's milk samples with levels ranging between 6.48 and 25.45 ng/L. Approximately 53.3% of the koumiss samples were found to contain AFM1, with levels ranging between 5.93 and 18.77 ng/L. None of the AFM1 levels in the tested samples exceeded above the 50 ng/L maximum limit set by the European Union for milk and the 500 ng/kg limit set by Kyrgyz Republic. As the levels of AFM1 in the raw mare's milk and koumiss were low, it can be said that these products do not pose a serious risk in terms of public health. This study has been an important source for determining AFM1 levels in mare's milk and koumiss.

Keywords: *Aflatoxin M$_1$, Mare's Milk, Koumiss, ELISA*

Kırgızistan Yaylalarında Üretilen Kısrak Sütü ve Kımız'ın AFM$_1$ Seviyelerinin Belirlenmesi

Öz

Kısrak sütü ve kımız, Orta Asya'da çok önemli iki temel gıda maddesidir. Bu çalışma, Kırgızistan'ın Narın, İssyk-Kul, Bişkek ve Talas bölgelerindeki yerel üreticilerden toplanan kısrak sütü ve kımız örneklerinde AFM1 kontaminasyonunu belirlemek amacıyla yapılmıştır. Bu amaçla Mayıs, Haziran ve Temmuz 2017'de toplanan 75 çiğ kısrak sütü ve 75 kımız numunesi ELISA yöntemi kullanılarak analiz edilmiştir. AFM1, 6.48 ile 25.45 ng/L arasında değişen seviyelerde 75 çiğ kısrak süt numunesinin %13.3'ünde tespit edilmiştir. Kımız numunelerinin yaklaşık %53.3'ünün AFM1 içerdiği bulunmuştur ve seviyeleri 5.93 ile 18.77 ng/L arasında değişmiştir. Test edilen numunelerdeki AFM1 seviyelerinin hiçbiri, Avrupa Birliği tarafından süt için belirlenen 50 ng/L maksimum sınırı ve Kırgıziztan tarafından belirlenen 500 ng/kg sınırını aşmadı. Çiğ kısrak sütü ve kımızdaki AFM1 seviyeleri düşük olduğu için bu ürünlerin halk sağlığı açısından ciddi bir risk oluşturmadığı söylenebilir. Bu çalışma kısrak sütü ve kımızdaki AFM1 seviyelerini belirlemesi bakımından önemli bir kaynak olmuştur.

Anahtar sözcükler: *Aflatoksin M$_1$, Kısrak Sütü, Kımız, ELISA*

INTRODUCTION

Mare's milk is one of the most important foods used since the ancient times, especially by the societies of central Asia and the former Soviet Union. Important differences exist among the mare, human and cow milk in terms of composition and nutritional properties [1,2]. Crude protein content of mare's milk is higher than human milk and lower than cow's milk. On the other hand, it is categorized in the group of albuminous milks since it contains about

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50% casein and about 40% whey protein in terms of protein content [2,3]. Thanks to its high content of whey proteins, mare's milk is rich in essential amino acids, with high biological value and true digestibility (95-97%) [4,5]. Its balanced amino acid content ensures healthy growth of human body [6]. Mare's milk contains less fat than human milk and cow's milk. Compared to the cow's milk, mare's milk is richer especially in linoleic (C18: 2) and linolenic (C18: 3) acids [2]. While mare's milk shows similarity to human milk in terms of lactose content, its lactose content is significantly higher than cow's milk. Its energy content is lower than both human milk and cow's milk [2,3]. This milk is a very good source of vitamin C (428 times more than cow's milk) [3]. When its composition and some structural features are considered, the mare's milk is more suitable for infant nutrition than cow's milk [2,3,7,8].

Koumiss is a traditional product that is produced by the fermentation of mare's milk and widely consumed in the central Asian countries. The high nutritional property of koumiss comes from the mare's milk used in its production. Koumiss is fermented by lactic acid bacteria, mainly including *Lactobacillus helveticus, Lactobacillus kefiranofaciens, Lactobacillus kefiri, Streptococcus parauberis, Lactococcus lactis*, and yeasts [9,10]. For hundreds of years, koumiss has been considered not only as a kind of food but also a natural and alternative medicinal remedy [11]. Koumiss consumption helps regulate the gut environment and immune system by modulating the intestinal microflora [12]. In some countries, koumiss has been successfully applied in combination with traditional medicine in treating clinical conditions like hepatitis, chronic gastric ulcer, and tuberculosis [10].

The basic structure of aflatoxins consists of bifuran and coumarin. The toxicity of aflatoxins comes from the bifuran structure and its carcinogenicity is due to coumarins [13-15]. Aflatoxins are an important global public health problem due to their naturally occurring toxic, carcinogenic, teratogenic and mutagenic effects in the feed and food industry [16]. Aflatoxins are basically toxic secondary metabolites produced by some Aspergillus species, such as *A. flavus, A. parasiticus*, and *A. nomius* Aflatoxin M_1 (AFM_1), a metabolite of aflatoxin B_1 and found in dairy products, is classified as a Group 1 toxin [16,17].

In order to protect consumers several countries have established legislation to regulate the levels of AFM_1 in milk and dairy products. These legal limits range from 50 ng/L in many countries such as those in the EU [18] to 500 ng/L in countries such as the US. The legal limit of AFM_1 levels in milk and milk products in Kyrgyz Republic is 500 ng/L [19].

AFM_1 contamination in raw milk is an important public health problem. The AFM_1 level has been examined in milk and dairy products in the World [15,20-23]. AFM_1 levels of milks obtained from different animals (buffalo, cow, goat, and sheep) [15,21,24] and different dairy products such as yoghurt, cheese, butter, and buttermilk are researched in the literature [23,25-28]. AFM_1 contamination has been reported even in breast milk from different countries [29]. Although mare's milk and koumiss are among the main foodstuffs of central Asian societies, no studies are found in the literature to report the AFM_1 contamination of these products.

The aim of this study was to investigate the AFM_1 content of raw mare's milk and koumiss obtained from milk producers located in the highlands of Naryn, Issyk-Kul, Bishkek and Talas in Kyrgyz Republic during milking season.

Materials and Methods

Sample Collection

A total of 75 raw mare's milk and 75 koumiss samples were collected from local producers in the highlands of Naryn, Issyk-Kul, Bishkek and Talas regions of Kyrgyz Republic. The samples were collected in May, June and July, 2017, when the mare's milk production is at its highest level, from 2 highlands in Talas (Bakai-Ata, Beshtash), 4 highlands in Bishkek (Kashka Suu, Chunkurchak, Kemin, Suusamyr), 2 highlands from Issyk-kul (Jeti Oguz, Ottuk), and 3 highlands from Naryn (At-Bashi, Song-Kul, Kochkor). These pastures are located at an altitude of 2.200 m above sea level. Pastures in this region are used for grazing by cattle, horses and small ruminants. The mares were on good quality pasture from spring to autumn without any concentrated feed supplement.

Measuring the AFM_1 Levels in the Raw Mare's Milk and Koumiss Samples

The AFM_1 levels in raw mare's milk and samples koumiss were determined using an enzyme-linked immunosorbent assay (ELISA) with a minimum detection limit of 5 ng/L (RIDASCREEN Aflatoxin M_1 test kit, R-Biopharm AG, Germany). AFM_1 standards (including 0, 5, 10, 20, 40, and 80 ng/L) are contained in the test kit. The test was used according to the manufacturer's instructions. A sample was considered to be negative for AFM_1 if the levels were below the minimum detection limit of the assay. The validation variables in this study were as follows:

Limit of detection (LOD) = 5 ng/L.

Limit of quantification (LOQ) = 8.5 ng/L.

Recovery rate = 100-116%, R.

Relative standard deviation calculated under repeatability conditions (RSDr) <10%.

Statistical Analysis

The study adopted statistical methods to evaluate the incidence of AFM_1 in samples, reported as mean and standard

deviation (SD). And the data were statistically analyzed by a one way analysis of variance (ANOVA) using SPSS version 19.0 (SPSS, Inc., Chicago, IL, US). The level of confidence required for significance was set at $P \leq 0.05$.

Results

In this study a total of 75 raw mare's milk samples and 75 koumiss samples were analyzed for AFM_1 with the ELISA. The occurrence and the distribution of AFM_1 concentration in various ranges in raw mare's milk and koumiss samples are presented in *Table 1*.

Table 1 shows the frequencies of AFM_1 contamination in the raw mare's milk samples from Bishkek, Issyk-kul, Naryn and Talas. Only three samples (12%) from Bishkek and two samples (11.1%) from Issyk-kul were found to be positive for AFM_1 contamination. However, the samples obtained from Naryn (15%) and Talas (16.6%) had a relatively high rate of contamination. The raw mare's milk samples with the lowest AFM_1 contamination rate were obtained from Issyk-kul (11.1%), which was followed by Bishkek (12%), Naryn (15%) and Talas (16.6%). From the 75 raw mare's milk samples 10 (n=13%) were found to be positive for AFM_1. Among these samples, the AFM_1 content in four of them (5.3%) was less than 10 ng/L, in five of them was between 10 and 20 ng/L, and in one (1.3%) ranged between 21 and 50 ng/L. The overall mean level of AFM_1 in the raw mare's milk samples was 13.39±5.87 ng/L. The minimum and maximum AFM_1 concentration in the raw mare's milk were 6.48 and 25.45 ng/L, respectively. None of the AFM_1 levels in the tested samples exceeded above the 50 ng/L maximum limit set by the EU [18] for milk and the 500 ng/kg limit set by Kyrgyz Republic [19].

The presence and concentration of AFM_1 contamination in the koumiss samples are presented in *Table 1*. A total of 15 samples (60 %) obtained from Bishkek, 11 samples (61.1%) from Issyk-kul, nine samples (45%) from Naryn and five samples from Talas were found to be positive for AFM_1. The koumiss samples with the highest AFM_1 contamination rate were obtained from Issyk-kul (61.1%), followed by Bishkek (60%), Naryn (45%) and Talas (41.6%). A total of 40 (53.3%) koumiss samples contained AFM_1. The minimum and maximum AFM_1 concentration in the koumiss samples were 5.93 and 18.77 ng/L, respectively. The average level of AFM_1 in the koumiss samples was determined as 8.38±3.14 ng /L. Furthermore, none of the koumiss samples contained AFM_1 concentrations above the highest tolerance limit (50 ng/L) set by the EU and Kyrgyz Republic (500 ng/L).

Discussion

Kyrgyz Republic is a northern Asian country where mare's milk and koumiss are widely produced and consumed. The producers settle in the highlands after mid-March. Therefore, milk and koumiss are produced when the mares are fed with fresh grass. Mare's milk is mostly produced in May, June and July. Kyrgyz use mare's milk for the production of drinking milk and koumiss. Although the positive effects of mare's milk and koumiss consumption on health are known, no study is found in the literature that reports the AFM_1 contamination of these products. For this reason, the findings of this study are believed to be important.

In recent times, high AFM_1 contamination reported in milk and dairy products in the world has become an important public health problem. Although no study exists in the literature on AFM content of raw mare's milk and koumiss, AFM_1 contamination has been reported in different milk types and fermented dairy products such as yogurt and ayran.

Table 1. *Occurrence and distribution of AFM1 contamination in raw mare's milk and koumiss samples collected from the highlands of Kyrgyz Republic*

Samples	Location	Highlands	Samples	Positive* (n/%)	Frequency Distribution of AFM_1 Concentration (ng/L)			AFM_1 Concentration (ng/L)			Above the EU** and Kyrgyz Republic Limit***
					< 10	10-20	21-50	Min	Max	Mean ± SD	
Mares milk	Bishkek	4	25	3 (12%)	1	1	1	8.68	25.45	16.66±8.41	0
	Issyk-kul	2	18	2 (11.1%)	1	1	-	9.73	13.48	11.61±2.65	0
	Naryn	3	20	3 (15%)	-	3	-	10.81	18.25	15.43±4.04	0
	Talas	2	12	2 (16.6%)	2	-	-	6.48	7.94	7.21±1.03	0
Total		11	75	10 (13.3%)	4 (5.3%)	5 (6.6%)	1 (1.3%)	6.48	25.45	13.39±5.87	0
Koumiss	Bishkek	4	25	15 (60%)	12	3	-	5.93	16.78	8.38±3.00	0
	Issyk-kul	2	18	11 (61.1%)	10	1	-	5.97	18.77	8.04±3.70	0
	Naryn	3	20	9 (45%)	8	1	-	6.56	14.31	7.73±0.88	0
	Talas	2	12	5 (41.6%)	4	1	-	5.99	16.33	8.99±4.32	0
Total		11	75	40 (53.3%)	34 (45.3%)	6 (8%)	-	5.93	18.77	8.38±3.14	0

** A sample was considered negative when its AFM_1 concentration did not exceed 5 ng/L, which was the detection limit of the RIDASCREEN Aflatoxin M_1 test kit; ** A sample was considered to be above the EU legal limit when its AFM_1 concentration exceeded 50 ng/L, *** A sample was considered to be above the Kyrgyz Republic legal limit when its AFM_1 concentration exceeded 500 ng/L*

Contrary to this study, high AFM_1 contamination in different milk types was reported in the literature. In one of the studies that report high AFM_1 contamination in milk samples [30] reported that all 110 samples were contaminated with AFM_1 between 0.028 and 4.98 µg/L, and 29 samples (26.3%) exceeded the legal limits (0.5 µg/L). Similarly, it was reported that 80% of milk samples sold in Syria are contaminated with AFM_1 and 52% of the milk samples exceed the EU's legal limits [31]. It was reported that 73.6% of 111 UHT milk samples and 131 pasteurized milk samples (n = 242) were contaminated with AFM at concentrations ranging from 100 ng/L to 22 ng/L, and 1.8% of UHT milk samples and 59.5% of pasteurized milk samples exceeded the EU's legal limits between 2016 and 2017 in China [32]. Similarly, in a three-year (2013-2015) study conducted in China, 1.8-11% of analyzed milk samples were found to be contaminated with AFM_1 above the EU's legal limits [33]. In a study conducted in Shush, Iran, it was reported that 75% of a total of 120 raw water buffalo milk and cow's milk samples are contaminated with AFM_1 and 8% of the samples exceed the EU's legal limits [34]. In a study conducted in Turkey, it was found that all 39 organic UHT milk samples (100%) exceeded the legal limit of Turkish Food Codex [35]. Also, high AFM_1 incidences have been reported in buffalo milk samples at 7.2%, 50%, 27%, 38.7% and 52% in Southern Italy, India, Afyonkarahisar of Turkey, Ahvaz of Iran, and Ismailia of Egypt, respectively [24,36-39].

Similarly, Hashemi [40] from Iran and Assem and Mohamad [41] from Lebanon, reported AFM_1 contamination in 56% and 65% of the milk samples, respectively, with 30% and 21% exceeding the legal limits set by EU. Differences in AFM_1 levels in various countries can potentially be attributed to geographical conditions, climate, animal feeding styles (pasture feeding, hay feeding, silage feeding), animal feed and dairy processing measures taken to prevent AFM_1 contamination. Therefore, when compared to the studies that report high level of AFM_1 contamination exceeding legal limits in Syria, China, Iran, Lebanon, and Turkey, the mare's milk produced in Kyrgyz Republic can be considered to be of very high quality. The low AFM_1 concentrations found in the mare's milk produced in Kyrgyz Republic show similarity to the studies conducted in China, Qatar, and Italy. It was reported that 5.650 raw milk samples collected during four seasons in 2016 from major dairies in China were contaminated with AFM at a rate of 4.7% and only 1.1% of the samples exceeded the EU's legal limits and AFM contamination incidence was at the highest level in winter [16]. Similar to this study, it was found in another study conducted in Southern China that 62.5% of raw buffalo milk samples were contaminated with AFM_1 between 4 and 243 ng/kg, and only 5.9% of samples exceeded the EU's legal limits. Also, it was reported that buffalo dairy products (HTST milk, UHT milk) were contaminated at concentrations between 10-50 ng/kg and the samples did not exceed the EU's legal limits [15]. In a study conducted in Qatar, it was found that 85% of milk samples are contaminated with AFM_1, but no sample exceeds the legal limits (50 ng/L) [25]. Likewise, the findings of two studies recently conducted in Italy can be evaluated as similar to the findings of this study. It was reported that 12% of the cow's milk samples analyzed in Northern Italy is contaminated with AFM_1 and only one sample exceeds the EU's legal limit [36] and in a study conducted in Bologna region [42], it was reported that none of the 60% positive milk samples exceeded the EU's legal limits.

In the literature, there are few studies reporting such low level of contamination. In this study, low concentrations of AFM found in raw mare's milk are quite striking. This may be due to the mares feeding fresh grass in the pastures. Because mares are milked mostly in May, June and July. During this period, the mares are fed with fresh grass in the pasture. Accordingly, studies have reported that the frequency of AFM_1 contamination is higher in winter months when the level of feeding with dry grass is high [16,43]. During the winter months, fresh grass consumption decreases, while stored concentrate feed consumption of animals increases. The risk of aflatoxin formation due to mold growth increases in the feeds that are stored under unsuitable conditions [44].

The AFM_1 concentrations found in the koumiss samples are very low. Although no studies exist on AFM_1 contamination in the literature, there are studies conducted on fermented dairy products such as yoghurt, ayran and laban. In a study conducted in Qatar, it was determined that 76% of the yoghurt samples and 76.1% of the laban samples were contaminated with AFM_1. However, the levels in none of the samples exceeded the legal limits (50 ng/L) [25].

In most studies, higher AFM_1 contamination is reported in fermented milk products such as koumiss. Contrary to the present study, it was determined that the yoghurt and ayran samples of a study conducted in Turkey contained 95% and 87.5% AFM_1, respectively, with 20% and 13.6% of these samples displaying levels higher than the maximum limit of 50 ng/kg determined by the Turkish Food Codex [27]. In a study conducted on organic yoghurt samples in Turkey, it was reported that all 26 samples exceeded the legal limit of Turkish Food Codex, which is 50ng/kg [35]. In their study conducted in Turkey [45] reported that 89% of the yogurt samples they tested contained AFM_1 and 26% of these samples had levels higher than the maximum limits set by the EU. Similarly, a study conducted in Pakistan reported the presence of AFM_1 in 61% of the yogurt samples with 47% of the samples having levels higher than the maximum limit of 50 ng/kg set by the EU [46]. It is found that, in 95% and 33% of the yogurt samples positive for AFM_1 contamination [47,48]. In a study conducted in the South of China, AFM_1 was detected in 55.5% of the yogurt samples, all of which had levels lower than 50 ng/kg [15].

The main reasons for detecting low concentrations of AFM_1 in koumiss may include the use of milk containing low

AFM_1 in production or it may be due to the fact that this is a fermented product. It is reported that the fermentation is effective in mycotoxin decontamination and reduction of mycotoxins, and is a feasible traditional food processing technique which does not only improve useful ingredients but also contributes to the food safety [49].

As the levels of AFM_1 in the raw mare's milk and koumiss were low, it can be said that these products do not pose a serious risk in terms of public health. In this study, although AFM_1 contamination in mare's milk and koumiss is below legal limits, especially the incidence of AFM_1, which is 53.3% in kumiss samples, should not be ignored due to the carcinogenicity of AFM_1.

Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

MAA, HÖ, FRİ and MA planned, designed, and supervised the research procedure. The samples were collected by FRİ. MAA and HÖ performed analysis. The manuscript was written by MAA and MA. MA contributed to the language editing of the final manuscript. All authors have interpreted the data, revised the manuscript for contents, and approved the final version.

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