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Evaluation of the Presence of Yeasts, Moulds and Aflatoxin M1 in Raw Sheep Milk in Mazandaran, Iran

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ABSTRACT

Introduction: Aflatoxins are among the most important fungal mycotoxins with carcinogenic properties. Aflatoxin M1 can be transmitted to humans through milk and dairy products. This study proposed to appraise the fungal and aflatoxin M1 contaminations in sheep milk collcted from different farms in Mazandaran province.

Methods: During July to September 2020, 70 samples of raw milk from sheep were collected in disparate regions of Northern Iran. All of the samples were analyzed in order to presence of fungal agents by culturing the raw milk onto Sabouraud dextrose agar (SDA) as well as AFM1 by competitive enzyme-linked immunosorbent assay (ELISA) technique.

Results: Of the fungal agents identified, the most frequently moulds were related to *Aspergillus* spp. (38.8%), *Cladosporium* spp. (22.4%) and *Penicillium* spp. (13.9%); and yeasts dependent on *Trichosporon* spp. (47.1 %), *Rhodotorula* spp. (33.8%) and *Candida* spp. (14.7%). *Of the 70 milk samples, all samples (100%) had* AFM1 *at condensation lower than the high level specified in European Union (EU) regulations, i.e., 50 ng/L.* The AFM1 contamination levels ranged from 1.15 to 48.50 ng/L with the average of 13.26 ± 4.87 ng/L.

Conclusions: Contamination of sheep milk with mycotoxins, especially AFM1, can be a potential risk for the consumer; therefore, raw sheep milk should be checked for these toxins.

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Introduction

Due to its nutrients, milk sheep can be a suitable environment for the growth of microorganisms. Fungal toxins are very harmful to health and economy. Mycotoxins can lead to the loss of protein and fat in sheep's milk, which spoils the milk and also annihilates their taste due to the production of lipase and protease enzymes (1). Toxic fungi are a concern for public health due to the production of mycotoxin, which is considered a secondary metabolite. Since Aflatoxins are toxic, teratogenic and having carcinogenic properties, they are considered as an undesirable material in milk and other dairy products (2). AFM1 appears as a major metabolite in the milk of ruminants when the animal consumes fodder contaminated with AFB1, which is produced by the activity of *Aspergillus parasiticus*, *Aspergillus flavus* and *Aspergillus nomius* in animal feed (3). AFB1 production occurs during harvest or during operation (post-harvest) as well as in storage following fungal contamination. Moreover, *Aspergillus* species amplify largely in tropical and subtropical climates which are under different environmental conditions (4). It is necessary to mention that in lactating animals, AFB1 is converted to AFM1 and excreted in milk by the hepatic microsomal hybrid oxidase system (5). Large and small ruminants are classified according to their ability to convert aflatoxin B1 to type M1. By examining the researches that have been done so far, this conversion ability has been reported for cows, goats and sheep as follows: 0.03 and 0.25 percent for sheep, 0.018 and 3.1 percent for goats and 0.2 to 6.2 percent for cattle (6). In general, goat and sheep milk are less contaminated with AFM1 in comparison to cow milk. On the other hand, for economic reasons, the use of concentrate and feedstuff in the feed formulation of small ruminants is limited (7).

Given the potential risk of AFM1, most countries have regulated AFM1 levels in milk and dairy products. These laws are often determined by

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considerations according to the economic conditions, so they may vary from country to country. For instance, the European Union (EU) accepts a maximum of 50 ng/L AFM1 in unprocessed milk (8). Most African, Asian, and Latin American countries have performed the mentioned measures; in addition, several studies have been conducted worldwide to determine AFM1 levels in cow's milk (9), buffalo milk (10), sheep milk and goat milk (11). So far, several studies have been accomplished on the presence of AFM1 in unprocessed cow milk in Iran (12-14), but in general there is not enough information about AFM1 in sheep milk and also, there is no data on yeast and mold contamination in sheep milk. In Mazandaran province, Iran, since a lot of milk is produced, sheep are the main sources of milk production in this region. Therefore, this study was conducted with the aim of investigating fungal contamination and aflatoxin M1 in sheep's milk from different farms in Mazandaran province.

Material and Methods

Raw milk collection

70 samples of raw sheep milk were received from 7 farms in Mazandaran province and it should be mentioned that (10 samples from each 7 farms) were collected in summer. The udder of each sheep was palpated before sampling to detect abnormal symptoms and abnormalities such as physical changes, asymmetry and swelling. After washing, each breast was dried with a special towel. In the next step, the teats were swapped with alcohol. Sterilized glass tubes with screw caps were used to collect 15-20 cc of milk from each quarter (it should be noted that the initial jets of milk were rejected). The samples were sent in a box containing ice for for immediate analysis. It should be noted that in case of any delay in evaluation, the samples were kept at -18°C. It should be considered that examinations and laboratory studies related to this study were performed in the mycology laboratory at the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Iran.

Mycological Evaluation

Evaluation of microbial properties including mold and yeast in sheep milk samples was performed based on the research by Spanamberg *et al*. (15) method. Briefly, 0.1 ml of the samples was streaked on Sabouraud dextrose agar using a plate technique. (SDA) (Merck Co., Darmstadt, Germany). The cultured plates were then incubated for 3-5 days at 30°C. Initially, yeasts were identified based on the physiological routine experiments (16) then differential tests like germ tube, chlamydoconidia production and culture were performed on differential Candida agar differential chromic culture medium. Arthroconidia-producing isolates were classified as *Geotrichum* or *Trichosporon*. In the next step, the number of fungal colonies was counted using the Protus Colony Counter (Synoptics, Cambridge, England) on the initial separation plate based on the Colony Formation Unit (CFU) and the results were announced per 0.1 ml of milk.

Measurement of AFM1 by ELISA Method

Here, competitive ELISA technique (Europroxima, Netherlands) was used to evaluate the level of AFM1 in raw milk samples. For this purpose, the samples were centrifuged at 4°C (2,000 × g) for 10 minutes. After centrifugation, two phases were formed. At the top, there was a layer of cream that was completely removed through a pasteurizer pipette, and 100 µl of the lower phase (degreased phase) was used directly in the test. 100 µl of the AFM1 standard solutions (0, 6.25, 12.5, 25, 50, 100 and 200 *ng/l*) and test samples (100 µl/well) were added to the microtiter plate wells and incubated for 60 minutes in the dark room temperature. After the washing steps, 100 µl of the enzyme conjugate was added and incubated for 60 minutes in dark room temperature. The washing steps had been repeated three times. In addition, fifty microliters of substrate and 50 µl of chromogen were added to each as well, mixed thoroughly and incubated for 30 minutes in the dark. Followed by the addition of 100 µl of the stop reagent to each well, the absorbance was measured at 450 nm in ELISA reader (ELX-800, Bio-Tek Instruments, USA).

Data Analysis

The statistical population was sheep milk samples collected. The study method is of laboratory type and the data obtained from the experiment was analyzed using SPSS Ver.22 software with utilization of (ONE-WAY ANOVA). Also, the season's effects on AFM1 pollution and Tukey comparison test was performed as well which at the end, a significant level of $P < 0.05$ was determined.

Results

As shown in Table 1, from milk samples collected from sheep farms in total yeasts: 68 CFU / 0.1 ml and molds: 129 CFU / 0.1 ml (a total of 197 fungal organisms. It should be noted that the difference between number of mold was significant (65.5%) and yeast (34.5%) in raw sheep milk (P <0.05) and thus in the following, explanations will be given related to the isolated fungal species will be described in the following: four out of 1, three out of 5, two out of 13, one out of 32 and zero out of 19 samples. Also, among different sheep farms, the average number of fungi varied between 1.2 CFU / 0.1 ml (on farms one and two) and 6.2 CFU / 0.1 ml milk (on farm six).

Table 1. Results of counting fungi in sheep milk samples collected from different farms in Mazandaran province (colony formation unit, CFU).

Farm	CFU/0.1 mL milk		
	Mean ± SD	Range	Result
1	1.2 ± 1.54	0-4	12
2	1.2 ± 0.97	0-3	12
3	3.3 ± 5.1	0-18	33
4	4.3 ± 5.47	0-16	43
5	2.0 ± 1.78	0-5	20
6	6.2 ± 6.24	0-20	62
7	1.5 ± 2.33	0-6	15

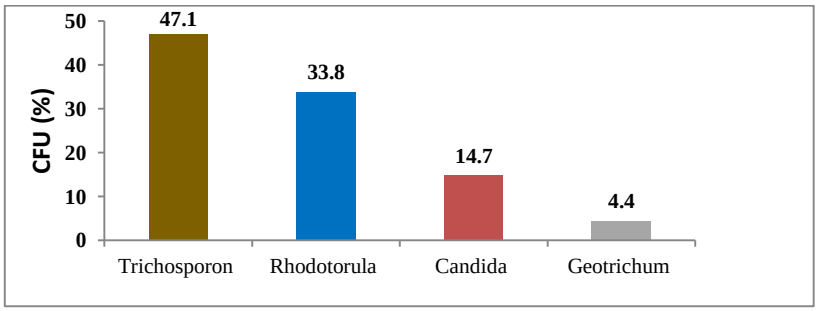

Figure 1. Comparative evaluation of different yeast species isolated from samples collected from different farms in Mazandaran province.

It should be noted that out of 70 evaluated samples, 14 species of fungal microorganisms belonging to 13 different genera were observed. Most yeasts isolated from the samples were associated with *Trichosporon* species. (no. 32, 47.1%), followed by *Rhodotorula* spp. (no. 23, 33.8%), *Candida* spp. (no. 10, 14.7%) and *Geotrichum* spp. (no. 3, 4.4%) (Figure 1). This study also found that *Aspergillus* spp. (no. 0, 38.8%), *Cladosporium* spp. (no. 29, 22.4%), *Penicillium* spp. (no. 18, 13.9%), *Fusarium* spp. (no. 14, 10.9%), Sterile hyphae (no. 8, 6.2%), *Scopolariopsis* spp. (no. 4, 3.1%), *Alternaria* spp. (no. 3, 2.3%), *Curvularia* spp. (no. 2, 1.6%) and *Paecilomyces* spp. (no. 1, 0.8%) as the most frequently isolated moulds. *Aspergillus fumigatus* (*A. fumigatus*) (28.7%) was found to be the most frequently isolated *Aspergillus* species, followed by *A. ochraceus* (8.5%) and *A. flavus* (1.6%) (Figure 2).

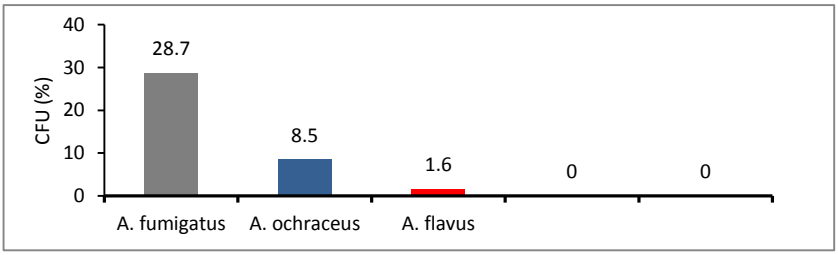

Figure 2. Comparative evaluation of *Aspergillus* species isolated from samples collected from different farms in Mazandaran province.

According to the 70 samples of raw sheep milk which were tested, all samples (100%) had AFM1 levels below the EU maximum (50 ng / l). The AFM1 contamination levels from 1.15 to 48.50 ng / l with 13.26 ± 4.87 ng / l (Mean ± SD concentration) are given in Table 2. Also, among the different samples collected, samples from farms 4 and 7 had the highest level of AFM1 contamination compared to other farms.

Table 2. Results from the observation of aflatoxin M1 in samples collected from different farms in Mazandaran province.

Farm	Season	AFM1 (ng/L)	
		Mean ± SD	Range
1	**Summer**	7.21 ± 4.02	3.32-15
2		6.27 ± 5.04	3.04-13.81
3		3.18 ± 0.66	1.15-3.92
4		21.78 ± 7.86	12.39-48.50
5		9.68 ± 3.89	5.25-14.7
6		12.83 ± 5.31	8.72-21.19
7		31.86 ± 7.33	17.4-42.53

Discussion

Although many studies have been conducted on milk and dairy products, limited information is available on indigenous communities living with raw milk. Fungi are known as a large microbial population in the milk of various animals, including sheep's milk. Present study demonstrated a total of 197 fungal organisms; indicating 65.5% moulds and 34.5% yeasts (P <0.05). Across several sheep farms, it was observed that the average number of fungi varied from 1.2 CFU / 0.1 ml of milk sample in the first and second fields to 6.2 CFU / 0.1 ml of milk sample in field number six. The differences in fungi number among the sampling sites (farms) display that the hygienic practices could create more significant role than geographic factors in this region.

In this study, 70 samples were fungi positive. It was detected that in most cases the isolated yeasts were associated with *Trichosporon* spp. (47.1%), followed by *Rhodotorula* spp. (33.8%), *Candida* spp. (14.7%) and *Geotrichum* spp. (4.4%). Based on the results obtained, Delavenne *et al*. (17) reported *Candida* spp., *Malassezia* spp., *Geotrichum* spp., *Rhodotorula* spp., *Debaryomyces* spp. and *Chrysosporium* spp. as the most commonly fungi in unprocessed sheep milk. According to a study by Spanamberg *et al*. (15), from raw sheep milk of *Geotrichum* spp., *Rhodotorula* spp., *Candida* spp., *Trichosporon* spp., together with *Cryptococcus* spp. and *Pichia* spp. were isolated. Panelli *et al*. (18) identified a total of 6 genera and 15 species of fungi in raw sheep milk. Among the 6 identified genera, *Geotrichum* spp., *Candida* spp., *Phaeosphaeriopsis* spp., *Pestalotiopsis* spp. and *Cladosporium* spp. belong to the phylum of Ascomycota, while *Cryptococcus* spp. was part of the phylum of Basidiomycota (19, 20). This study also discovered *Aspergillus* spp. (38.8%), *Cladosporium* spp. (22.4%), *Penicillium* spp. (13.9%), *Fusarium* spp. (10.9%), sterile hyphae (6.2%), *Scopolariopsis* spp. (3.1%), *Alternaria* spp. (2.3%), *Curvularia* spp. (1.6%) and *Paecilomyces* spp. (0.8%) as the most frequently isolated moulds. *A. fumigatus* (28.7%) was detected to be the most frequently isolated *Aspergillus* species, followed by *A. ochraceus* (8.5%) and *A. flavus* (1.6%). These findings are consistent with previous studies on the fungal diversity of sheep, cow, and goat milk. Discrepancies in fungal diversity among the variety of animal species and sampling periods can be explained by animal's environment and physiological state (21). Delavenne *et al*. (17) showed that moulds belonging to genera *Fusarium* (20% of positive samples), *Cladosporium* (10%), *Aspergillus* (10%) and *Penicillium* (5%) were isolated from sheep milk. These genera, together with yeasts, have been commonly celibate from sheep milk as well as from the teat surface, silage, dust and air from farms (20, 21) which are therefore the most probable sources of milk contamination. The composition of yeasts and molds in raw milk and dairy products can vary depending on various factors, including health measures as well as geographical conditions (22).

In this study, all samples (100%) had AFM1 levels below the EU maximum (50 ng / l). AFM1 contamination levels ranged from 1.15 ng / l to 48.50 ng / l with a mean concentration of 13.26 ± 4.87 ng / l. Existing differences can be due to varieties in storage time and conditions, as well as diversity in feed sources on farms. Screening

of untreated cow's milk in many various regions of Iran was performed to determine AFM1 (12-14). However, Limited studies have been performed to investigate the level of AFM1 contamination in the raw milk of Iranian sheep. In a research which has done credibly by Movassagh (23), AFM1 was found in 30% of raw sheep milk samples with concentrations less than 5 *ng/L* in Tabriz city.

In all samples examined, the contaminated samples did not exceed the maximum limit set for AFM1 by the European Union (EU). In a recent study from Kermanshah, Ilam and Hamadan, AFM1 contamination regarding to the raw sheep milk (5.7-82 ng/l) was 65.3% and in 26.9% sheep milk samples were above the EU limit (24). In general, there is not enough information available worldwide about the presence of aflatoxin M1 in fresh sheep milk.

The AFM1 levels found in this study appear to be in line with those in other studies carried out in some European countries, which revealed a low frequency of AFM1 as well as very low-toxin levels in positive samples (25, 26). In accordance with our findings, 99.3% of sheep milk samples were below the EU limit in a study which performed in Spain (27). Bognanno *et al*. (28) detected AFM1 at low level (1-10 ng/L) in 79% of Italian sheep milk samples and ranging from 10 to 50 ng/l in the 20% of the samples and only three samples were above the legal limits (50 ng/l). Similar results were stated by Virdis *et al*. (29) with an average concentration of 12.59±14.05 ng/l, ranging from 8.72 to 58.82 ng/l in Italy. In Croatia, AFM1 levels in sheep milk were discovered during the period July–September 2013, and ranged from 2.11 to 40.8 ng/l (30). Similar low AFM1 levels in sheep milk, with mean values of 3.3 and 6.85 ng/l, were reported in Lebanon (31). In Punjab, city of Pakistan, in all samples examined, the contaminated sample did not overstep the maximum limit set for AFM1 by the EU (32). Various reasons such as farm management, food diversity, country and geographical area, season plus analytical methods have changed the level of AFM1 reported in various studies (33). One of the reasons for the lower level of aflatoxin M1 contamination in sheep's milk compared to cow's milk (29) this is because the sheep consumes less concentrate and mainly grazes in open pastures (34). In addition, lower carry-over rates were found in dairy sheep, ranging between 0.03 and 0.25% in dairy cows comparison (0.2 and 6.2%) (6). Farm type has a significant effect on AFM1 contamination. Sheep milk samples were collected from farms 4 and 7, which had the highest AFM1 contamination compared to other farms. The reason for this seems to be that sheep are probably fed more concentrate feeds. It should be noted that the probability of growth of toxic species of Aspergillus and subsequent production of aflatoxin B1 in animal feed that is stored in the warm season and in adverse conditions is high (35).

Conclusions

Food safety control organizations should pay attention to fungal contamination as well as the level of aflatoxin M1 in the qualitative evaluation of sheep milk. So far, no study has been conducted in Mazandaran province on the amount of aflatoxin M1 in sheep's milk, and this study was conducted for this purpose. From our study, it was found raw sheep milk fungal agents variations with Aspergillus and Trichosporon species predominance. Also, in all samples tested, the concentration of aflatoxin M1 were lower than the maximum limit set (50 ng /l) by the European Union. In addition, continuous and accurate inspection and control of milk as well as sheep feed for the presence of fungi and aflatoxins is very important for proper health management. The first effective step in reducing the amount of aflatoxin M1 in sheep milk is to improve the methods of storing and preparing animal feed.

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Conflict of Interests

There is no conflict of interests in this study.

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