Estimation of Aflatoxin M1 Levels in Some Dairy Products Manufactured from Raw Milk Experimentally Inoculated with Toxin

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Summary

Fate of AflatoxinM1 in soft white cheese and its by-product (whey) and in yogurt locally made from raw sheep's and goat's milk experimentally inoculated with 0.05 and 0.5 µg/l AflatoxinM1 were investigated using ELISA technique. Results reported that AflatoxinM1 was concentrated in cheese at levels significantly higher than that recorded in the raw milk that used for its processing, with a significant decrease in AflatoxinM1 levels in its by-product (whey) comparable to the raw milk used in manufacturing at both inoculated levels. Yogurt produced from raw sheep's milk at second inoculated level exerted AflatoxinM1concentration significantly lower than that present in the milk. Significant differences in AflatoxinM1distribution in cheese and whey produced from sheep's milk comparable to their counterparts produced from goat's milk were recorded. Finally, results revealed the efficacious role of the various dairy manufacturing processes in AflatoxinM1 distribution and the necessity to issue of local legislations concerning the maximum permissible limits for AflatoxinM1 in milk in order to stay within the universal permissible levels for AflatoxinM1 in dairy products to provide greater protection for consumer health.

Keywords: AflatoxinM1, ELISA technique, soft white cheese, whey, yogurt.

Introduction

Milk is considered as one of the most important nutrients that humans need in their food system at all stages of life. Milk is a complete diet as it contains many vital nutrients useful to the body. Also it is a rich source of many vitamins and minerals necessary for the body. Many dairy products can be manufactured from milk, which are considered as essential sources of nutrients present in milk, which may outperform milk in their nutritional value. These products are produced by several methods depending on the type of product and the source of the milk from which they are made (1). Despite the extensive prevalence and large global consumption of bovine milk and its products at present for its availability and the length of its production season, both sheep's and goat's milk are still very popular and are used in the manufacture of many dairy products that have distinctive qualities and are universally desirable, especially cheese and yogurt. Recent researches conducted at the last years were increased the popularity of goat's milk. Many research show the benefits of goat's milk, its products, and drinks rich with this milk type for human health and growth. Also goat's milk can be digested easily and contains less amounts of allergens compared to other milk types (2 and 3). In Iraq, soft white cheese (Arab cheese) usually made from raw sheep's or goat's milk and yogurt especially made from sheep's milk, which is locally known as (Allaban alkhathir), are the most popular foods on breakfast tables, due to their palatable taste and easy digestion, in addition to their high nutritional value. These products are particularly prevalent in the markets in the spring. With regard to whey (the by-product of the cheese industry), it has been introduced in several food industries in many countries at recent decades because of its high nutritional value and the characteristics of its ingredients (4).

Aflatoxins are one of the highly toxic mycotoxins, which are secondary metabolites produced by certain molds, especially Aspergillus flavus and A. parasiticus, in addition to A. nomius, A. tamarii, A. pseudotamarii, A. ochraceoroseus and A. bombycis (5 and 6). There are four main types of aflatoxins: B1, B2, G1 and G2. Aflatoxin B1(AFB1) is the most toxic (7). When feeding lactating animals on AFB1 contaminated feedstuffs, and as a result of metabolic processes within the liver, AFB1 will be converted to AFM1, the hydroxylated metabolite of AFB1, which is excreted in milk (8). Milk contaminated with AFM1 and dairy products manufactured from it are the major sources of human exposure to AFM1 (9). The risk from human exposure to AFM1 arises from its direct toxic effects on the cells of the body without the need to be subjected to metabolic processes inside the body to show its toxic effects as with AFB1. Also AFM1 has carcinogenic and mutagenic effects (6). Therefore, many countries set strict legislations on the maximum permissible limits for AFM1 in milk 0.05 μg/l and in cheese 0.25 μg/kg in accordance with European legislation and 0.5 μg/l in milk according to the U. S. Food and Drug Administration (10-12).

Presence of AFM1 in dairy products may be return to the presence of toxin residues in raw milk of lactating animals that have already been fed on AFB1contaminated feeds. In addition, the usage of powdered milk or some food additives contaminated with AFM1 for manufacturing of dairy products (13). Due to the presence of AFM1 in the milk and dairy products constitutes a serious public health problem and there is no previous studies related to the fate of AFM1 in locally processed dairy products in Nineveh Governorate, therefore, the current study aimed to estimate the levels of AFM1 in locally prepared soft white cheese (Arab cheese) and yogurt (Allaban), which were manufactured from experimentally inoculated raw sheep's and goat's milk with the maximum allowable limits for AFM1 in milk (0.05 and 0.5 μg/l) according to European and American determinants, respectively.

Materials and Methods

Raw sheep's and goat's milk samples were provided from some animals´ owners in Nineveh Governorate, Iraq. The samples were put in sterile polyethylene bags and immediately transported to the laboratory in a refrigerated containers (4˚C) for processing of dairy products under study. Raw sheep's and goat's milk were mixed well and samples from each milk type were taken in duplicate for AflatoxinM1 (AFM1) estimation prior to milk inoculation with the toxin. Raw milk of sheep and goats were divided into two groups for each milk type (4 kg/ group) and inoculated with 0.05 and 0.5 µg/l AFM1 (Sigma-Aldrich, Germany) after thoroughly mixing. Samples from each group of milk were taken in duplicate for AFM1 determination after mixing well with the toxin and the remaining were subjected for manufacturing dairy products under study.

Raw sheep's and goat's milk experimentally inoculated with 0.05 and 0.5 µg/l AFM1 were used for processing of dairy products under study. Soft white cheese was prepared according to the procedure proposed by (14). Yogurt was processed according to the method mentioned by (15). Representative samples from soft white cheese, whey and yogurt were taken in duplicate .

Competitive Enzyme Linked Immuno sorbent Assay (ELISA) technique was used for quantitative analysis of AFM1 in raw sheep's and goat's milk and their products according to the procedure proposed by AFM1 kit manufacturer (RIDASCREEN® Aflatoxin M1 30/15, R-Biopharm, Darmstadt, Germany). Raw milk samples of sheep and goats and their products were prepared according to the method described by RIDASCREEN kit(16). Samples of raw milk and whey were centrifuged at 3500 rpm for 10 min. at 10 °C. The upper cream layer was completely removed and 100 µl from the skimmed milk (defatted supernatant) was used directly to conduct the competitive ELISA test.

Yogurt samples were pasteurized by heating them to 80 °C for 3 min. (17). The samples were cooled to room temperature and 10 g from each sample was diluted with 100 ml of PBS (pH 7.2). The mixture was homogenized by stirring, and 100 µl from diluted yogurt samples were taken for AFM1 estimation.Two grams from well homogenized cheese samples were taken and 40 ml of dichloromethane was added for each of them. The extraction was done by shaking the mixture for 15 min. The suspension was filtered and 10 ml from the filtrate was evaporated. The residue was dissolved in 0.5 ml of methanol, 0.5 ml of phosphate buffer saline (PBS) and 1 ml of n-heptane. The mixture was centrifuged at 2700 rpm for 15 min. The upper heptane layer was completely removed, and 100 µl from the aliquot was diluted with 400 µl from the kit buffer. The diluted samples (100 µl each) were used in the test.

ELISA test was performed according to the kit manufacturer's instructions. One hundred microliters of the standard solutions of AFM1 (0, 5, 10, 20, 40, 80 ng/l) and previously prepared samples were put into separate wells of the microliter plate (in duplicate) and incubated for 30 min. in the dark at room temperature (22-25°C). The liquid was poured out, and wells were washed twice with the washing buffer (250 µl) using an ELISA washer (ELX-50, Bio-Tek®, USA). Then, one hundred microliters of the diluted enzyme conjugate was added for each well, mixed gently and incubated for 15 min. in the dark at room temperature. The contents of the wells were poured out and each well was washed again with the washing buffer (250 µl) three times. After that, 100 µl of the substrate and the chromogen were added for each well, mixed gently and incubated for 15 min. in the dark at room temperature. Finally, 100 µl of the stop solution was added for each well with gently mixing. Reading was performed at a wave length of 450 nm using an ELISA reader (ELX-800, Bio-Tek®, USA) within 15 min. RIDA® SOFT WIN program prepared by R-Biopharm AG, Germany was adopted for results processing.

The results were statistically analyzed using Two Way Analysis of Variance procedure of Sigma Stat for windows Version 3.10 (2004) (18). The significant differences ($p<0.05$) between mean concentrations of AFM1 were detected based on Duncan's Multiple Range Test (19).

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Results and Discussion

Results related to estimation of AFM1 levels in soft white cheese and its by-product (whey) and in yogurt produced from raw milk of sheep and goats experimentally inoculated with AFM1 at a concentration of 0.05 µg/l showed a significant increase ($p<0.05$) in the mean concentrations of AFM1 in soft white cheese produced from raw sheep's and goat's milk (0.113 and 0.0944 µg/kg) respectively, compared with raw milk (0.051 µg/l), where they recorded AFM1 concentration factors in cheese of 2.22 and 1.85 times, respectively higher than in raw milk. In turn, this increment led to a significant decrease ($p<0.05$) in the mean concentrations of AFM1 in its by-product (whey) (0.0129 and 0.0310 µg/l) respectively, where they recorded percentages of AFM1 reduction 74.71% and 39.22%, and AFM1 remaining in whey 25.29% and 60.78%, respectively. These results are consistent with the results of other studies, which indicate that AFM1 is more concentrated in cheese than in whey, but they differ from them in the AFM1 concentration factor in cheese and percentage of AFM1 remaining in whey, as in the study presented by (20) which clarified that AFM1 concentration in the cheese curd made from raw sheep's milk inoculated with AFM1 at 0.05 µg/l reached to 2.7 times higher than that reported in the milk used for manufacturing, while the percentage of AFM1 remaining in whey reached to 42.3% of the initial concentration.

In addition to the results reported by (21) in which they pointed out that AFM1 was concentrated in the soft cheese produced from milk naturally polluted with AFM1 at a concentration of 0.055 µg/l to 1.87 times higher than that reported in the milk used for processing. The affinity of AFM1 for casein protein in cheese is greater than its affinity for whey proteins because it is a semi-polar component. Furthermore, the high dry matter content in cheese, especially casein protein, may explain the reasons of AFM1 concentration in cheese in many studies which ranged between 2-5 times higher than that reported in the milk used for manufacturing (22-25). There is a significant variation in the percentages of AFM1 remaining in whey. Some researchers (26-28) reported that about half the amount of AFM1 or more in milk is remained in the whey (66%, 53-58%, 100%), respectively. Other researchers indicated that the percentages of AFM1 remaining in whey ranged between 40-60% according to the results recorded by (29) and Deveci (24), and may be higher than that 70-74% (23), or much less 23.6-38.5% (25). The reason for this significant variation in the percentages of AFM1 remaining in whey may belong to several factors, including quality of milk used, degree and type of pollution in the milk used in the manufacture, whether natural, in which the levels of AFM1 are usually lower than those reported in the experimentally contaminated milk, type of cheese produced, techniques used in its manufacture, possibility for presence of small pieces of curd in the whey, and techniques used in the measurement (24,27, 29 and 30). Results showed a decrease in the concentrations of AFM1 in yogurt produced from raw milk for sheep and goats 0.0502 and 0.0508 µg /kg, respectively that not significantly different from those recorded in the raw milk, with the percentages of AFM1 reduction 1.57% and 0.39%, and AFM1 remaining in yogurt 98.43% and 99.61%, respectively.

The results of other studies differed about the levels of AFM1 in the yogurt produced from milk contaminated with the toxin. Several studies have indicated a reduction in the levels of AFM1 in the yogurt, as in the study conducted by (31), which indicated a decrease in AFM1 levels at the end of the fermentation period to 13 and 22% in the yogurt with pH values of 4.6 and 4, respectively, which processed from milk experimentally toxin inoculated. Also the study recorded by (32), which showed a gradual reduction in the levels of AFM1 during the fermentation process to 13, 22 and 35% after 6, 24 and 48 hours of fermentation, respectively. In addition to the study reported by (21), in which AFM1 reduction level reached to 5% in fresh yogurt prepared from milk naturally contaminated with AFM1 at a concentration of 0.055 µg/l. Whereas other studies (33 and 34) indicated that the level of AFM1 in the yogurt was stable and not affected by its manufacturing processes. While some studies reported an increase in the level of AFM1 in the yogurt, as in the studies presented by (35 and 36). Regardless of the raw milk type used, results showed the important role played by the various dairy manufacturing processes in the distribution of AFM1 in dairy products under study when using milk contaminated with the toxin to manufacture these products. Results showed a significant increase ($p<0.05$) in the mean concentration of AFM1 in soft white cheese prepared from raw milk experimentally inoculated with 0.05 µg/l AFM1 (0.104 µg/kg), which resulted in a significant decrease ($p<0.05$) in the mean concentration of AFM1 in whey (0.0220 µg/l) compared with the raw milk (0.0510 µg/l).

Yogurt recorded a decrease in the mean concentration of AFM1 (0.0505 µg/kg) which not significantly different from that in the raw milk. When comparing the results of AFM1that present in the samples of soft white cheese, whey and yogurt produced from raw sheep's milk with those produced from raw goat's milk, it was noted that the increment in the mean concentration of AFM1 was significantly higher ($p<0.05$) in cheese made from raw sheep's milk (0.113 µg/kg) compared to that made from the raw goat's milk (0.0944 µg/kg), which resulting in a reduction in the mean concentration of AFM1 in whey obtained from cheese produced from raw sheep's milk (0.0129 µg/l) significantly higher ($p<0.05$) than that recorded in whey obtained from cheese produced from raw goats' milk (0.0310 µg/l).

The differences in the proportions of milk components among different animal species, especially with regard to protein, specifically casein, may play an important role in the distribution of AFM1, especially during cheese manufacturing (37). There was no a significant difference ($p<0.05$) in concentrations of AFM1 between yogurt produced from raw sheep's milk (0.0502 µg/kg) compared to that produced from raw goat's milk (0.0508 µg/kg) (Table,1and 3).With regard to samples of soft white cheese, whey and yogurt produced from raw milk of sheep and goats experimentally inoculated with 0.5 µg/l AFM1, as shown in (Table, 2 and 3), a significant increase ($p<0.05$) in the mean concentrations of AFM1 in cheese processed from raw sheep's and goat's milk (1.071 and 0.852 µg/kg) respectively, compared to those recorded in the raw milk (0.496 and 0.498 µg/l) respectively, with concentration factors for AFM1 2.16 and 1.71 times higher than in the raw milk. As a result, a significant decrease ($p<0.05$) was reported in the mean concentrations of AFM1 in whey obtained from cheese produced from raw sheep's and goats' milk (0.136 and 0.325 µg /l, respectively), where the percentages of AFM1 reduction 72.58% and 34.74% and AFM1 remaining in whey 27.42% and 65.26%, respectively. The pattern of AFM1 distribution between cheese and its by-product (whey) may return to the affinity of AFM1 for casein protein mainly concentrated in cheese, in addition to the high dry matter content in cheese, specifically for casein (22-25). Results revealed a significant decrease ($p<0.05$) in the mean concentration of AFM1 in yogurt produced from raw milk of sheep (0.466 µg/kg) compared with the raw milk, where the percentage of reduction in AFM1 reached to 6.05% and the percentage of AFM1 remaining in yogurt 93.95%. These results are in line with the results of other studies indicating reduction of AFM1 levels in yogurt (21,31 and 32).

Several factors may play an important role in the reduction of AFM1 levels in yogurt. The low pH in yogurt will change the structure of milk proteins, which in turn may effect on the association of AFM1 with these proteins. Formation of organic acids or other by-products of fermentation process especially lactic acid during yogurt manufacturing may remove the toxin. In addition to existence of some strains of lactic acid bacteria which have the ability to bind to AFM1 causing reduction of free toxin content during yogurt producing. Also the enzymatic, microbial, and in particular acid fermentation may lead to degradation of the toxin (37-39). Whereas yogurt prepared from raw goat's milk showed a decrease in the mean concentration of AFM1 (0.480 µg/kg) that not significantly different ($P<0.05$) from that present in the raw milk, where the percentage of AFM1 reduction was 3.61% and AFM1 remaining in yogurt 96.39%.

Regarding the role of different dairy manufacturing processes in the distribution of AFM1 when the type of raw milk used was not taken into consideration, results showed a significant increase ($p<0.05$) in the mean concentration of AFM1 in soft white cheese prepared from raw milk experimentally spiked with 0.5 µg/l AFM1 (0.961 µg/kg), which resulted in a significant decrease ($p<0.05$) in the mean concentration of AFM1 in whey (0.230 µg/l) compared with the raw milk (0.497 µg/l). Yogurt also reported a significant decrease ($p<0.05$) in the mean concentration of AFM1 (0.473 µg/kg) compared with the raw milk.

Comparison of AFM1 existence in soft white cheese, whey and yogurt samples manufactured from raw sheep's milk with those manufactured from raw goat's milk showed that the increment in AFM1 concentration in cheese produced from raw sheep's milk (1.071 µg/kg) was significantly higher ($p<0.05$) than that recorded in cheese produced from raw goat's milk (0.852 µg/kg), which led to a decrease in AFM1 concentration in whey obtained from cheese made from raw sheep's milk (0.136 µg/l) significantly higher ($p<0.05$) than that reported in whey obtained from cheese made from raw goat's milk (0.325 µg/l). These results were attributed to the variation in milk constituent's proportions between different animal species as it was previously mentioned (37). Results did not show a significant difference ($p<0.05$) in the reduction in the mean concentrations of AFM1 between yogurt manufactured from raw sheep's milk (0.466 µg/kg) with that manufactured from raw goat's milk (0.480 µg/kg).

From our results data we conclude that soft white cheese may constitute the most threatening product for public health as accommodating the largest proportion of AFM1 when prepared from milk contaminated with the toxin compared with yogurt and whey. Because there is no legislations concerning the upper permissible limits for AFM1 in milk and dairy products in Iraq, therefore it is necessary at present to adopt the European legislation concerning the upper allowable limits for AFM1 in raw milk to reach to the safe levels of the toxin in the dairy products under study when prepared locally for keeping the health of the consumer.

Table, 1: Mean concentration of AFM1 (µg/kg or l) in soft white cheese, whey and yogurt produced from raw sheep's and goat's milk experimentally inoculated with AFM1 at a concentration of 0.05 µg/l .

Raw milk type	Raw milk µg/l	Soft white cheese µg/kg	Whey µg/l	Yogurt µg/kg
Sheep	0.0510±0.00078 a	0.00138±0.113 b	0.00079±0.0129 d	0.00025±0.0502 a
Goat	0.0510±0.00063 a	0.0944±0.00064 c	0.0310±0.00024 e	0.0508±0.00045 a
Effect of dairy processing	0.0510±0.00048 A	0.104±0.00285 B	0.0220±0.00277 C	0.0505±0.00026 A

*Horizontally different small letters are significantly different (p<0.05) .
*Vertically different small letters are significantly different (p<0.05) .
*Different capital letters within last raw are significantly different (p<0.05).

Table, 2: Mean concentration of AFM1 (µg/kg or l) in soft white cheese, whey and yogurt produced from raw sheep's and goat's milk experimentally inoculated with AFM1 at a concentration of 0.5 µg/l.

Raw milk type	Raw milk µg/l	Soft white cheese µg/kg	Whey µg/l	Yogurt µg/kg
Sheep	0.496±0.00089 a	1.071±0.0180 b	0.136±0.0103 d	0.466±0.00082 f
Goat	0.498±0.00068 a	0.852±0.0147 c	0.325±0.00543 e	0.480±0.00066 af
Effect of dairy processing	0.497±0.00062 A	0.961±0.0349 B	0.230±0.0291 C	0.473±0.00215 D

*Horizontally different small letters are significantly different (p<0.05) .
*Vertically different small letters are significantly different (p<0.05) .
*Different capital letters within last raw are significantly different (p<0.05).

Table, 3: Concentration coefficient of AFM1 in soft white cheese and the percentages of AFM1 reduction and AFM1 remaining in both whey and yogurt manufactured from raw sheep's and goat's milk at both inoculated levels (0.05 and 0.5 µg/l).

Inoculated level	Raw milk type	Cheese	Whey		Yogurt	
		Concentration coefficient	% AFM1 reduction	% AFM1 remaining	% AFM1 reduction	% AFM1 remaining
0.05	Sheep	2.22	74.71	25.29	1.57	98.43
	Goat	1.85	39.22	60.78	0.39	99.61
0.5	Sheep	2.16	72.58	27.42	6.05	93.95
	Goat	1.71	34.74	65.26	3.61	96.39

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تقدير مستويات سم الافلا M1 في بعض منتجات الالبان المصنعة من حليب خام مطعم تجريبيا بالسم

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الخلاصة

تناولت الدراسة التحري عن مصير سم الافلا M1 في كل من الجبن الأبيض الطري وناتجه العرضي (الشرش) وفي الزبادي المصنعين محليا من الحليب الخام للأغنام والماعز المطعم تجريبيا بسم الافلا M1 بتركيز 0.05 و 0.5 مايكروغرام /لتر باستخدام تقنية الأليزا. سجلت النتائج ارتفاعا معنويا في معدل تركيز سم الأفلا M1 في الجبن مقارنة بالحليب الخام الذي صنع منه، والذي أدى بدوره الى حصول انخفاض معنوي في معدل تركيز السم في ناتجه العرضي (الشرش) مقارنة بالحليب الخام عند كلا مستويي الحقن. أظهر الزبادي المصنع من حليب الأغنام الخام عند المستوى الثاني للحقن انخفاضا معنويا في معدل تركيز السم مقارنة بالحليب الخام. سجلت النتائج وجود فروقات معنوية في توزيع سم الأفلا M1 في الجبن وناتجه العرضي (الشرش) المصنعين من حليب الأغنام الخام بالمقارنة مع مثيلاتها المصنعة من حليب الماعز الخام. وقد خرجت النتائج بأهمية الدور الذي تلعبه العمليات التصنيعية المختلفة للألبان في توزيع سم الأفلا M1، وبضرورة اصدار التشريعات المحلية المتعلقة بالحدود العليا المسموح بها لسم الأفلا M1 في الحليب من أجل عدم تجاوز المستويات العالمية المسموح بها للسم في منتجات الألبان من أجل توفير حماية أكبر لصحة المستهلك.

الكلمات المفتاحية: سم الأفلا M1، تقنية الاليزا، الجبن الأبيض الطري، الشرش، الزبادي.