Assessment of Aflatoxin M₁ Concentrations During Production and Long Storage of Salted (Tuzlu) Yogurt

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Abstract: In this survey, it was aimed to determine the concentration of aflatoxin M₁ (AFM₁) in milk during the production and storage of salted (tuzlu) yogurt using High Performance Liquid Chromatography (HPLC). Salted (tuzlu) yogurt was produced artificially from contaminated milk with AFM₁ at two different levels (0.05 µg/l and 0.1 µg/l). Yogurt and strained yogurt production caused losses of AFM₁ about 65%, 70.25% and 73.75%, 81.12% respectively, in milk contaminated with 0.05 µg/l AFM₁ and 0.1 µg/l AFM₁. Also, it was determined that the storage process of the salted (tuzlu) yogurt (90 days) decreases the AFM₁ content of the salted (tuzlu) yogurt by 0.019 and 0.027 µg/l (0.05 µg/l and 0.1 µg/l AFM₁ respectively). Difference among dates in storage period was found to be statistically significant (P<0.01). Salted (tuzlu) yogurt has long shelf life and high heat processing, and AFM₁ is not completely lost in both levels.

Keywords: Aflatoxin M₁, HPLC, Salted (tuzlu) yogurt, Stability, Storage

1. Introduction

Aflatoxins are synthesized, especially by the Aspergillus parasiticus, Aspergillusnomius and Aspergillus flavus species [1,2], and rarely by other Aspergillus, Penicillium and Rhizopus species [3,4]. Up to now, almost 19 different toxic differentiation of aflatoxins have been declared [5]. A. parasiticus produces both B and G aflatoxins, while Aspergillus flavus only produces B aflatoxins [6]. International agency for research on cancer (IARC) categorized AFB₁ as Group I of carcinogen and AFM₁ as Group 2B of carcinogenic compounds [1,7].

AFB₁ is thought to be the most potent toxic aflatoxin and metabolically produces the monohydroxy derivative AFM₁ [8,9,10]. AFM₁ is almost as acutely toxic as AFB₁, while its mutagenic and carcinogenic potential seems to be lower [11,12,13]. Aflatoxins metabolized to the 8-9-epoxide connect macromolecules and cause cancer, hepatopathy and immunosuppression [9,14].

The United States Food and Drug Administration has defined a limit of 500 ng/l for AFM₁ in milk and dairy products [15], while the European Commission has defined a limit of 50 ng/l for AFM₁ in these products.

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[16]. The Turkish Food Codex legal limits for AFM$_1$ in milk is 0.05 µg/kg [17]. Aflatoxins cause cancer, slow down child development, suppress the immune system, and may cause death [18]. Therefore, it is significant to assessment of aflatoxin M$_1$ concentrations in milk and dairy products since it poses a potential health hazard.

The most significant problem caused by milk and dairy products in terms of AFM$_1$ is that it is stable against heat process such as UHT, sterilization and pasteurization. This is the reason why AFM$_1$ does not decrease in amount during the manufacture of dairy products [19]. Salted (tuzlu) yogurt, which is prepared by heating (second pasteurized at 90°C) of strained yogurt, is a traditional milk product that has a high amount of dry matter and long shelf-life [20,21]. 1.5% of salt is added into the salted (tuzlu) yogurt during the heating process to eliminate microbial development, and to decrease water activity [22,23,24].

The aim of this study was to investigate the dispersion and stability of AFM$_1$ during the manufacture and the storage of salted (tuzlu) yogurt. In this study, by adding AFM$_1$ in two doses (0.05 µg/l and 0.1 µg/l) into the milk used for producing salted (tuzlu) yogurt, the effects of straining, heat treatments (applied to milk and strained yogurt) and storage on the change of the initial concentration of AFM$_1$ were investigated. The changes in the AFM$_1$ content during manufacturing and storage were determined by the immunoaffinity column, High Pressure Liquid Chromatographic method.

2. Material and Method

2.1. Experimental design and preparation of salted (tuzlu) yogurt samples

During the manufacturing of the salted (tuzlu) yogurt, a total of 24 liters of raw cow milk was used. The milk was divided into three equal 8 liter measurements. A standard aflatoxin M$_1$ (Sigma-Aldrich, CAS 6795-23-9; C17H12O7; FW 238.3; Co., 3050 Spruce Street, MO 63103, St. Louis, USA) was spiked to raw milk at the levels of 0.05 µg/l (A) and 0.1 µg/l (B). The last measurement of milk was taken as the control group (C) and no aflatoxin M$_1$ was added. After the samples were pasteurized at 95°C for 20 min, the 3 groups of milk were cooled to 43±1°C [23]. The yogurt culture was inoculated into the milk (2.5%) (YO-MIX, Real 500 and 600 series, DANISCO), and the samples were incubated at 42±1°C for 2.5-3 hours at pH 4.7. The yogurt samples were cooled by keeping them at room temperature for 15 min. Then, whey of the yogurt was drained. The strained yogurt was pasteurized at 90°C for 90 min (second pasteurization). At this point, salt was added about 1 g/100 g to sample A and B. The samples were cooled to 20°C. The salted (tuzlu) yogurt samples were placed into plastic-originated vacuum bags [25]. The samples were then transferred into a refrigerator, and stored at 4°C for 90 days (Figure 1). The samples were analyzed at storage days 1, 30, 60, and 90, at 4°C. All the analyses were replicated three times.

![Figure 1. Schematic diagram of the manufacture of salted (tuzlu) yogurt](image)

2.2. Aflatoxin M$_1$ analyses

The aflatoxin M$_1$ analysis was realized by High Performance Liquid Chromatography (HPLC) and using an immuno-affinity column (Afla M$_1$ HPLC, Vicam, USA) [26]. The AFM$_1$ standard was supplied from the Sigma company (Sigma-Aldrich, CAS 6795-23-9; C17H12O7; FW 238.3; Co., 3050 Spruce Street, MO 63103, St. Louis, USA), and prepared according to Anonymous [26]. The aflatoxin M$_1$ concentrations of samples was determined in Shimadzu HPLC system. C18 Lichrospher column (25x4.6 mm, 5 µm, Waters Spherisorb ODS-2, Germany) were used as analytical columns. The chromatographic separation composition was carried out using a fluorescence detector (an excitation wavelength of 360 nm and an emission wavelength of 430 nm) with a mobile phase (at a flow rate of 1 ml / min) containing acetonitrile: water (25:75, v / v). Under these circumstances the AFM$_1$ was eluted from the column at around 5 minutes.

The pure aflatoxin M$_1$ standard in a crystal form was dissolved in chloroform to prepare the stock solution. A series of calibration solutions were prepared at different concentrations (µg /ml) AFM$_1$ using the prepared stock solution (Figure 2). Calibration curves are arranged by plotting the peak area for each calibration solution against the mass of injected AFM$_1$. The detection limit of AFM$_1$ was 0.01 µg/kg. Their AFM$_1$ contents were calculated and the recovery of the AFM$_1$ was found to be 99.72%. The analytical results were not corrected for the recovery (Figure 2).
Sample preparation: The milk samples were heated to 37°C, and then filtered through Whatman No 4 filter paper [27]. The filtered milk (50 ml) was passed through an immuno-affinity column (3 ml/min). After, 1.25 ml methanol: acetonitrile (20:30) was collected in a vial by passing it through a column. 100 µl of prepared vial content was injected into the HPLC.

This process was conducted by modifying the method given by Govaris et al. [25], and Martins and Martins [28]. The yogurt samples were homogenized by stirring, and a 20 g sample was weighed. Chloroform (75 ml), saturated NaCl solution (1 ml) and diatome soil (5 g) were homogenized at a high speed for 2-3 minutes. Hexane (50 ml), distillated water (30 ml) and methanol (1 ml) were added into the evaporated sample. The bottom phase was passed through an immuno-affinity column (3 ml/min). 100 µl of prepared vial content was injected into the HPLC.

2.3. Statistical analyses

The obtained data were evaluated by the variance (ANOVA). The Tukey Tukey’s multiple range test was used in the general linear model of the SPSS statistical package (SPSS 15.0 SPSS Ltd. Working UK) test was applied to see the difference between the samples. The differences between the averages were regarded significant at P<0.05 and P<0.01.

3. Results

It was determined that there was 0.047 and 0.098 µg AFM1 per liter of pasteurized milk respectively (Table 1). In this study, it was found that 0.05 µg/l AFM1 (A) and 0.1 µg/l AFM1 (B) added to the milk decreased to 0.020 µg and to 0.034 µg through the yogurt production. AFM1 was not detected in the sample C (Control). After the filtration of yogurt serum, the AFM1 content of the strained yogurt samples (A and B) was found to be 0.030 µg and 0.043 µg, respectively. AFM1 was determined as 0.005 µg and 0.012 µg in the serum of yogurt. To emphasize the AFM1 losses, the total AFM1 content of the raw milk contaminated with AFM1 was considered as 100%. The total aflatoxin M1 losses in the products produced from this contaminated milk were shown in Table 1. In this study, it was determined that the AFM1 content in the strained yogurt was higher than the yogurt samples, due to an increase of the dry matter.

In fermentation of yogurt, pH decreases, organic acids and some other fermentation by-products (such as volatile fatty acids, amino acids, peptides or aldehydes) occur. These compounds formed in yogurt and decreased pH may cause a reduction in the amount of AFM1 [29]. In addition, it is reported that the lactic acid bacteria used in fermentation reduce the amount of AFM1. In a recent study, AFM1 binding ability of lactic acid bacteria (Lactobacillus plantarum, Lactobacillus helveticus and Lactococcus lactis) and Saccharomyces cerevisiae strains were investigated in milk samples containing AFM1 at concentrations of 0.05 µg/l and 0.1 µg/l. As a result of these research, Lactobacillus helveticus and Saccharomyces cerevisiae strains were found to be 100% bound to AFM1. In addition, it was determined that Lactobacillus helveticus had higher binding potential than other lactic acid bacteria [30]. Some researchers reported levels of AFM1 in four milk samples ranging from 53.7 to 123.8 ng/kg were found to exceed the EU MRL of 50 ng/kg, whereas levels of AFM1 in 214 samples of processed UHT milk ranged from 2.29 to 21.4 ng/kg were found to all below the LOQ value [31]. In another recent study from China also reports AFM1 content of UHT milk samples in 2014 and 2015 found to be 88.6% and 59.6%, respectively [32]. AFM1 in the milk is comparatively stable, and it is not exterminated by pasteurization or heat treatments, therefore causes a serious health risk [10].

Nadira et al. [33] declared that 4/53 of dairy product samples had the contamination level greater than the European Commission (EC) limit (>50 ng/l). Iqbal and Asi [34] reported that AFM1 was detected in 61% of yogurt samples. Approximately 47% of these yogurt samples were found above the EU recommended limit. A recent study in Iran also reported that the rate of cow milk and cheese samples exceeding the EU limit were 35.9% and 10%, respectively and also explained that there is a relationship between the season and aflatoxin M1 content [35]. The reason for the decrease of the AFM1 content after production of the yogurt could be based on a low pH, by-products of fermentation or lactic acid fermentation 

Figure 2. Chromatograms of AFM1 and the calibration curve of AFM1

| AFM1 Concentration (ng/ml) | Retention Time (min) | R² Value |
|---------------------------|---------------------|----------|
| 0.012                     | 0.009               | 0.968    |
| 0.020                     | 0.015               | 0.986    |

Table 1. Concentrations During Production and Long Storage of Salted (Tuzlu) Yogurt
acid bacteria and organic acids. The change in the structure of the casein during the yogurt production and the by-products occurring after the fermentation should be prevented for the safe effective in manufacturing. Strained yogurt during pasteurization and salting of pasteurized milk, yogurt, strained yogurt produced from 0.05 and 0.1g/kg AFM$_1$ contaminated milk (n=3)

| Samples                  | Sample amount (kg) | Concentrations of AFM$_1$ (µg/l) | Total AFM$_1$ (µg) | AFM$_1$ content during manufacture (%) |
|--------------------------|--------------------|----------------------------------|--------------------|----------------------------------------|
| Raw milk                 | 8.0                | 0.05 ± 0.001                     | 0.1 ± 0.001        | 0.4                                     | 100                                   |
| Pasteurized milk         | 8.0                | 0.047 ± 0.003                    | 0.098 ± 0.001      | 0.376                                  | 94                                    |
| Yogurt                   | 7.0                | 0.020 ± 0.003                    | 0.034 ± 0.013      | 0.140                                  | 95                                    |
| Strained yogurt          | 3.5                | 0.030 ± 0.007                    | 0.043 ± 0.001      | 0.105                                  | 26.25                                 |
| Serum                    | 3.5                | 0.005±0.001                      | 0.012±0.001        | 0.017                                  | 4.25                                  |

A: 0.05 µg/l AFM$_1$ added salted (tuzlu) yogurt, B: 0.1 µg/l AFM$_1$ added salted (tuzlu) yogurt. *The total AFM$_1$ content of 0.05 and 0.1 µg/l AFM$_1$ added milks to be 100%.

Table 2. Effect of storage period on the AFM$_1$ levels of salted (tuzlu) yogurt produced from 0.05 and 0.1g/kg AFM$_1$ contaminated milk (n=3)

| Salted (tuzlu) yogurt samples* | Concentrations of AFM$_1$ (µg/l) | Total AFM$_1$ (µg) | AFM$_1$ content during manufacture (%)** |
|--------------------------------|----------------------------------|--------------------|----------------------------------------|
| 1st day                        | 0.062 ± 0.002$^a$               | 0.044 ± 0.002$^a$ | 0.045                                  | 11.25                                 |
| 30th day                       | 0.026 ± 0.000$^a$               | 0.034 ± 0.006$^a$ | 0.046                                  | 11.5                                  |
| 60th day                       | 0.022 ± 0.003$^a$               | 0.027 ± 0.006$^a$ | 0.039                                  | 8.5                                   |
| 90th day                       | 0.019 ± 0.003$^a$               | 0.027 ± 0.003$^a$ | 0.034                                  | 9.5                                   |

A: 0.05 µg/l AFM$_1$ added salted (tuzlu) yogurt, B: 0.1 µg/l AFM$_1$ added salted (tuzlu) yogurt. a, b: Difference among dates in storage period has been found to be statistically significant (P<0.01). *The amount of salted (tuzlu) yogurt samples are 1.78 kg. **The total AFM$_1$ content of 0.05 and 0.1 µg/l AFM$_1$ added milks to be 100%.

A decrease in the aflatoxin concentration has also been defined in some acidified milk [36]. Hassanin [37] reported lactic acid that develops in yogurt during fermentation could cause the degradation of AFM$_1$. The AFM$_1$ levels of yogurt samples were found to be 0.043 and 0.075 µg/l, respectively and these AFM$_1$ values become 0.052 and 0.088 µg/l after filtration have been reported by Gоварис et al. [25].

It was determined that AFM$_1$ content became 0.026 µg at 30th day and 0.022 µg on the 60th day in salted (tuzlu) yogurt (A). Also, the AFM$_1$ content of the sample A was reported to decrease to 0.019 µg in 90 days of storage. It was found that the AFM$_1$ content of salted (tuzlu) yogurt (B) was 0.034 µg/l, 0.027 µg/l, 0.027 µg/l on the 30th, 60th, 90th day, respectively. The difference among samples was found to be significant statistically (P<0.01) (Table 2).

Iha et al. [38] decated that the effects on AFM$_1$ of yogurt production and storage were minimal and total AFM$_1$ mass in milk was reduced by 6% in yogurt. Another research that aflatoxin M$_1$ in yogurt was reduced to around 59% of the level in milk during refrigerated storage at 4°C [37]. During the production and storage of yoghurt, changes in aflatoxin M$_1$ levels may be caused by factors such as the pH, the concentrations of aflatoxin M$_1$ in the milk [39]. The other most important reason for that decrease may be second pasteurized and salting of strained yogurt during salted (tuzlu) yogurt manufacturing.

Motawee [40] reported that the losses of AFM$_1$ were 20.5%, 21.4%, 22% for cheese curd prepared with 6%, 8% and 10% salt. However, the salt ratio (about 1%) of salted (tuzlu) yogurt is lower than cheese curd. It is believed that the effect on amount of AFM$_1$ of salt ratio is very little.

4. Discussion and Conclusion

In this study, two different levels of AFM$_1$ (0.05 µg/l and 0.1 µg/l) were added in to milk during the manufacturing process of salted (tuzlu) yogurt. It was found to decrease the initial amount in both two concentration levels in all of the samples. This indicates that the production phases of the salted (tuzlu) yoghurt and the 90 day storage decreased the initial AFM$_1$ contents. The factors that are effective in the reduction of AFM$_1$ are on the following: pasteurizing the milk, the filtering of the yogurt serum, the pasteurizing of the yogurt. However, even though some processes such as heat treatment (first pasteurization at 95°C for 20 min in milk and second pasteurization at 90°C for 90 min in strained yoghurt), salt addition (about 1 g/100 g) in the production of salted (tuzlu) yogurt and 90 days of storage had been carried out, none of the samples had been completely removed from the AFM$_1$. According to these findings, contamination should be prevented for the safe consumption of milk and milk products.

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