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RESEARCH ARTICLE

Effects of Pasteurization and Storage on Stability of Aflatoxin M1 in Yogurt [1]

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Summary

In present study yogurt was produced from cow's milk contaminated artificially with aflatoxin M_1 (AFM_1) at two different levels, 1.5 and 3.5 μg kg^{-1} (ppb), and the effects of pasteurization and storage on the AFM_1 contents were investigated. It was found statistically important that pasteurization at 95°C for 5 min caused losses of AFM_1 in milk contaminated with 1.5 and 3.5 μg kg^{-1} AFM_1 about 18% and 16% , respectively ($P<0.01$). After yogurt production, total AFM_1 levels of yogurt samples produced by 1.5 and 3.5 μg kg^{-1} AFM_1 contaminated milk samples were decreased to 36.5 and 34.6%, respectively. After the 2-week refrigerated storage at 4°C, AFM_1 content of yogurts produced from 1.5 and 3.5 μg kg^{-1} AFM_1 contaminated milks decreased by 6.5 and 9.0%, respectively.

Keywords: *Aflatoxin M_1, Milk, Stability, Yogurt, Storage*

Yoğurtta Aflatoksin M1 Stabilitesi Üzerine Pastörizasyon ve Depolamanın Etkileri

Özet

Bu çalışmada aflatoksin M_1 (AFM_1) ile iki farklı düzeyde, 1.5 ve 3.5 μg kg^{-1} (ppb), doğal olarak kontamine olmuş inek sütünden yoğurt üretilmiş ve AFM_1 içerikleri üzerine pastörizasyon ve depolamanın etkileri araştırılmıştır. 95°C'de 5 dakika pastörizasyonun, 1.5 ve 3.5 μg kg^{-1} AFM_1 ile kontamine sütte AFM_1'de sırasıyla %18 ve %16 oranında istatistiksel açıdan önemli bulunan kayba neden olduğu bulunmuştur ($P<0.01$). Yoğurt üretiminden sonra, 1.5 ve 3.5 μg kg^{-1} AFM_1 ile kontamine sütten üretilen yoğurt örneklerinin toplam AFM_1 düzeylerinde sırasıyla %36.5 ve %34.6 oranlarında düşüş olmuştur. Buzdolabında 4°C'de 2 haftalık depolamanın ardından 1.5 ve 3.5 μg kg^{-1} AFM_1 ile kontamine sütten üretilen yoğurt örneklerinin toplam AFM_1 içerikleri sırasıyla %6.5 ve %9.0 oranında azalmıştır.

Anahtar sözcükler: *Aflatoksin M_1, Süt, Stabilite, Yoğurt, Depolama*

INTRODUCTION

Aflatoxins are highly toxic, mutagenic, teratogenic and carcinogenic compounds that have been implicated as causative agent in human hepatic and exrahepatic carcinogenesis [1,2]. AFM_1 found in animal milk is a major metabolite of aflatoxin B_1 (AFB_1) produced by *Aspergillus flavus* and *Aspergillus parasiticus* which contaminate agricultural commodities and feeds [3]. A direct relationship has been observed between the amount of AFM_1 in milk and AFB_1 consumption via feedstuffs. The conservation rate of ingested AFB_1 into AFM_1 is highly variable, ranging from 0.3% to 6.2% [1-3].

AFM_1, initially classified as a Group 2B agent (International Agency for Research on Cancer, IARC), has now moved to Group 1 carcinogenic agent by IARC [4]. Therefore, the presence of AFM_1 in milk and milk products is considered to be undesirable [5,6]. The occurrence of AFM_1 in milk and milk products is one of the most serious problems for food safety because of its importance as a foodstuff for adults and especially children [5-7].

It is of interest to know how the processes common to the dairy industry influence the AFM_1 content of milk and milk products. There are some contradictions in literature relating this issue. Some studies indicate that pasteurization

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and sterilization treatments are not reduce the content of AFM_1 in milk [5,6,8]. However, some reports show that content of AFM_1 are degraded depending on time and temperature combination of heat treatment applied [9,10]. Similarly, Fallah [4] reported that AFM_1 is relatively stable during processing and storage of various dairy products.

The objective of the present work was to determine the stability of AFM_1 during production and storage of yogurt. For that purpose, the milk used for yogurt production was artificially contaminated with AFM_1 at the level of 1.5 and 3.5 µg kg^{-1} and two batches of yogurt containing AFM_1 at two different levels were obtained. Effects of process stages such as heat treatment, incubation and cold storage on AFM_1 stability were investigated.

MATERIAL and METHODS

Production of Yogurt Samples Contaminated with AFM_1

Whole cow milk (6 kg) obtained from the Ankara University Agricultural Faculty dairy farm was equally divided into four portions. Two of 1.5 kg portions were spiked with standard AFM_1 (Sigma Chemical Co. St. Louis, USA) at the levels of 1.5 µg kg^{-1} while other two 1.5 kg portions were spiked with 3.5 µg kg^{-1} of AFM_1. Milk was sampled for AFM_1 analysis before and after the spiking procedure. Contaminated milks were pasteurized at 95°C for 5 min, cooled to 4°C and then 2% of mixed culture (1:1 ratio of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, YC 380, Direct Vat Set type, Chr. Hansen A/S. Horlom, Denmark) were added. After the shaking to distribute culture evenly each 1.5 kg milk portions were fermented at 43°C until 4.6 of pH. Following incubation process, yogurt samples were cooled to 4°C. With this production method four yogurt samples containing AFM_1 at two different concentrations (1.5 and 3.5 µg kg^{-1}) were produced. Two of them (contaminated with 1.5 and 3.5 µg $kg^{-1}$of AFM_1) were used for analysis while other two samples were stored at 4°C for two weeks.

Analysis of Milk and Yogurt Samples for AFM_1

Milk samples (100 mL) were warmed to 37°C under gentle stirring for better fat dissolution. Samples were subsequently centrifuged at 4.000 rpm for 10 min and the upper thin fat layer was discarded (if any). The bottom skimmed milk layer was filtered through Whatman 4 paper (Brendford, UK) and 50 mL filtrate was collected for analysis.

Yogurt samples were prepared according to Mayes and MacDonalds [12]. Briefly, yogurt (40 g), Celite-545 (10 g), chloroform (150 mL) and saturated sodium chloride solution (2 mL) were mixed by Ultra Turrax (Junke and Kunkel, GmbH, Germany) at medium speed for 2 to 3 min. The slurry was filtered through Whatman 4 filter paper. Filtrate was evaporated to dryness under vacuum at 60°C using rotary evaporator (Heidolph Rotary Evaporator vv-2000, Germany). Two milliliters of methanol and 60 mL of phosphate-buffered saline (900 mL water, 8 g NaCl, 1.16 g Na_2HPO_4 mixed, and pH was adjusted to 7.4) were added to the residue, and the mixture was placed into the separating funnel (500 mL). Then, hexane (100 mL) was added, and mixture was shaken vigorously and the layers were allowed to separate and a portion of the bottom (aqueous) layer (50 mL) was collected for analysis.

An aliquot (50 mL) of the filtrate from each sample (milk and yogurt) was passed through an immunoaffinity column (Vicam AFLA M_1, USA) containing specific monoclonal antibodies bound to a solid support material. As the sample passed through the column, the antibodies selectively bound with AFM_1 present and formed an antibody-antigen complex. Other components of the sample matrix were washed off the column with 10 mL of water. The toxin was slowly eluted from the column with 3 mL of acetonitrile, and final extract was evaporated to 300 µL under the nitrogen stream and finally was redissolved in the HPLC (High performance liquid chromatography) mobile phase to 3 mL again. The volume of injection into HPLC was 100 µL. AFM_1 chromatogram of milk sample contaminated artificially with AFM_1 at the level of 1.5 µg kg^{-1} was given in *Fig. 1*.

A liquid chromatographic system (Model 1100, Hewlett Packard, USA) equipped with a Octadodecil silicagel C-18 (5µm, 250 x 4.6 mm i.d.) column (Hicrom, Reading, UK) was used for AFM_1 determination. The mobile phase consisted of acetonitrile-water (25:75, v/v) was delivered to the column at a rate of 1 mL min^{-1}. Under these conditions AFM_1 was eluted from the column at 10.5 min. Fluorescence detector (Model G1321A, Hewlett Packard, USA) was used with excitation wavelength of 360 nm and an emission wavelength of 430 nm.

Preparation of Calibration Solutions for AFM_1

Pure, AFM_1 standard (Sigma-Aldrich, USA) was dissolved in chloroform to prepare a stock solution. A series of calibration solutions were prepared using stock solution in the concentrations of 0.5, 1.0, 2.5, 5.0 and 7.5 AFM_1 ng mL^{-1}. Calibration curves were constructed by plotting the peak area for each calibration solution against the mass of AFM_1 injected. The

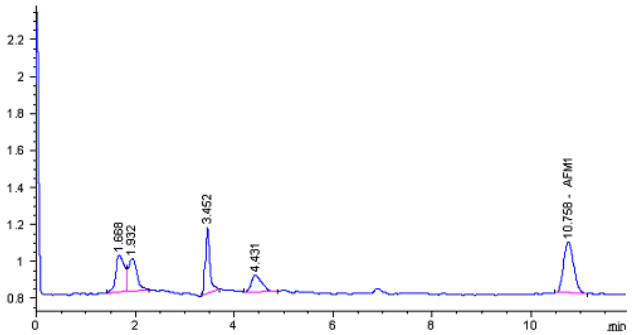

Fig 1. AFM_1 chromatogram of milk sample contaminated artificially with AFM_1 at the level of 1.5 µg kg^{-1}

Şekil 1. 1.5 µg kg^{-1} düzeyinde AFM_1 ilave edilmiş süt örneğine ait kromatogram

detection limit was 0.01 µg kg^{-1}. Recoveries of AFM_1 from milk and yogurt were 99.60% and 94.42%, respectively. Analytical results were not corrected for recovery.

Statistical Analysis

Data were subjected to analysis of variance, whereas differences between means were tested for significance by Duncan's multiple range test in the general linear model of SPSS statistical package (SPSS version 10.0., SPSS Ltd. Working, UK). Differences between means were considered significant at P<0.01.

RESULTS

Total AFM_1 content of raw milk contaminated with 1.5 and 3.5 µg kg^{-1} of AFM_1 (RM-1.5 and RM-3.5), pasteurized milk and yogurt produced from those contaminated raw milks are shown in *Table 1*. Total AFM_1 of RM-1.5 was 2.175 µg. After pasteurization at 95°C for 5 min, it decreased to 1.785 µg. In other words, heating of milk at 95°C for 5 min caused a significant (P<0.01) loss of AFM_1 at the level of 17.93%. Similarly, a decrease in AFM_1 content was observed on subjecting the RM-3.5 to pasteurization at 95°C for 5 min. After pasteurization, the AFM_1 level of RM-3.5 decreased to 4.250 µg from those initially present in milk at the level of 5.063 µg. This 16.06% decrease was also found to be significant (P<0.01).

Total AFM_1 of yogurt samples contaminated with 1.5 and 3.5 µg kg^{-1} of AFM_1 (YOG-1.5 and YOG-3.5) produced from RM-1.5 and RM-3.5 were 1.381 µg kg^{-1} and 3.312 µg kg^{-1}, respectively. This indicated a significant decrease (P<0.01) from those initially presented in milk at the ratio of 36.51% for YOG-1.5 and 34.58% for YOG-3.5.

The concentration of AFM_1 in yogurt did not remain constant during the 2-week refrigerated storage period

Table 1. *Total AFM_1 contents of samples contaminated with 1.5 and 3.5 µg kg^{-1} AFM_1*

Tablo 1. *1.5 ve 3.5 µg kg^{-1} AFM_1 ile kontamine örneklerin toplam AFM_1 içerikleri*

Samples	Sample Amount	AFM_1 (µg kg^{-1})	Total AFM_1 (µg)	Loss of Total AFM_1 (%)[c]
Contaminated with 1.5 µg kg^{-1} AFM_1				
RM	1.500 kg	1.450±0.010	2.175[a]	-
PM	1.500 kg	1.190±0.027	1.785[b]	17.93
YOG	1.490 kg	0.927±0.068	1.381[c]	36.51
Contaminated with 3.5 µg kg^{-1} AFM_1				
RM	1.500 kg	3.375±0.038	5.063[a]	-
PM	1.500 kg	2.833±0.052	4.250[b]	16.06
YOG	1.490 kg	2.223±0.018	3.312[c]	34.58

RM: *raw milk,* ***PM:*** *pasteurized milk,* ***YOG:*** *yogurt, Sample weight was taken into consideration in the calculation of AFM_1 loss, [a,b] Differences between the averages of sample with different capital letters in same column were significant (P<0.01), [c] Loses in percentage is calculated according to AFM_1 level of RM*

Table 2. *Effect of storage period on the AFM_1 levels of yogurts samples produced from 1.5 and 3.5 µg kg^{-1} AFM_1 contaminated raw milk*

Tablo 2. *1.5 ve 3.5 µg kg^{-1} AFM_1 ile kontamine çiğ sütten üretilen yoğurt örneklerinin AFM_1 içerikleri üzerine depolamanın etkisi*

Samples	Sample Amount	AFM_1 (µg kg^{-1})	Total AFM_1 (µg)	Loss of Total AFM_1 (%)[c]
Contaminated with 1.5 µg kg^{-1} AFM_1				
YOG	1.490 kg	0.927±0.068	1.381[a]	100
YOG after 2-week of storage	1.490 kg	0.867±0.024	1.292[a]	6.45
Contaminated with 3.5 µg kg^{-1} AFM_1				
YOG	1.490 kg	2.223±0.018	3.312[b]	100
YOG after 2-week of storage	1.490 kg	2.023±0.026	3.014[b]	9

YOG: *Yogurt. Sample weight was taken into consideration in the calculation of AFM_1 loss, [a,b] Differences between the averages of sample with different capital letters in same column were significant (P<0.01), [c] Loses in percentage is calculated according to AFM_1 level of RM*

(Table 2). Throughout the storage period 6.45% decrease was observed in the total AFM_1 content of YOG-1.5 which was found significant (P>0.01). Similarly, when the total AFM_1 content of YOG-3.5 was considered as 100%, its AFM_1 level decreased to 91% after 2-week storage. This 9% decrease was not found significant (P>0.01).

DISCUSSION

In this study, results showed that pasteurization process during yogurt production had an effect in the way of decrease of AFM_1. This decrease may be attributed to simultaneous interactions of whey proteins with casein, which may hinder the extraction of casein which is bounded with aflatoxin M_1 and consequently prevent heat destruction of the toxin. Wiseman et al.[14] were also implied that changes in AFM_1 stability by heat treatment could be the result of changed extraction of the toxin after milk was heated and it might be showing an effect of extraction efficiency than a real decrease in AFM_1 content.

Results of milk samples are in agreement with those of El-Deep et al.[9], who found 9.5% and 26% decreases in AFM_1 after heat treatment at 63°C for 30 min and at 121°C for 15 min, respectively, while Kiermeier and Mashaley [13], reported that various heat-time treatments caused reductions in the AFM_1 concentrations of milk varying from 12 to 40%. Choudhary et al.[10] have recently studied the effect of various heat treatments on AFM_1 stability of cow's milk and reported that sterilization of milk at 121°C for 15 min caused 12.21% degradation of AFM_1, whereas boiling decreased AFM_1 by 14.50%. They also concluded that destruction of AFM_1 depends on time and temperature combination of the heat treatment applied. However, some reports showed that aflatoxin was stable during heat-treatments such as pasteurization and sterilization [8,14].

Also, the results of this study were similar to obtained from Megalla and Hafez [15] in a study on the effect of yogurt production on AFB_1. They reported complete removal of 800 µg kg^{-1} of AFB_1 by its transformation into $AFB_{2\alpha}$ and concluded that the acidity of yogurt was responsible for this conversion. Rasic et al.[16] reported that AFB_1 levels decreased from 90-97% after fermentation. Similarly, Hassanin [17] mentioned a 17% decrease of AFM_1 in yogurt whereas Govaris et al.[8] determined that AFM_1 levels in yogurt samples showed a significant decrease after fermentation. On the contrary, Blanco et al.[18] reported that the yogurt production has no influence on AFM_1 while Munksgaard et al.[19] showed a slight increase in concentration of AFM_1 in yogurt from those initially presented in raw milk.

The decrease of AFM_1 during yogurt production might be attributed to factors such as low pH, formation of organic acids or other fermentation by-products and presence of lactic acid bacteria [20]. Changes in pH due to the acid development can affect the structure of milk proteins. Changes in casein structure may also affect the relationship between AFM_1 and casein [21]. In a study conducted by Govaris et al.[20], AFM_1 was significantly lower ($P<0.01$) in yogurts with pH 4.0 than in yogurts with pH 4.6. Production of larger amounts of lactic acid and other fermentation by-products along with the lower pH could cause this further decrease of AFM_1. Lactic acid and several other fermentation by-products such as volatile fatty acids, amino acids, peptides and aldehydes could also cause degradation of AFM_1 in yogurt [20-22]. Rasic et al.[16] showed a significant decrease of AFB_1 in milk acidified with lactic, citric or acetic acid. It was also found that lactic acid bacteria either viable or heat-killed could reduce the amount of AFB_1 and AFM_1 [24]. Govaris et al.[20] reported that the growth rate of *Streptococcus thermophilus* was affected by the higher level of AFM_1 but *Lactobacillus delbrueckii* subsp. *bulgaricus* was not affected by the presence of AFB_1 (600 - 1400 µg kg^{-1}) in milk during fermentation of yogurt.

Blanco et al.[18] reported that AFM_1 in yogurt was stable until 7th day of storage but at the end of the 12th day, AFM_1 content decreased at the level of 9%. Hassanin [17] also reported 40 and 59% of AFM_1 loss in yogurt samples after the 7 and 13 days of storage at 4°C, respectively. Whereas, Govaris et al.[21] determined that storage of yogurt for 2 and 4 week resulted in 15.6 and 16.2% of AFM_1 lost, respectively.

Results indicated that the initial amount of AFM_1 in milk decreased after yogurt production. Some factors such as low pH, presence of lactic acid bacteria, organic acids and fermentation by products, could responsible for this decrease. Throughout the storage period of 2 weeks AFM_1 contents of yogurt samples were almost stable. Briefly, when the milk contaminated with AFM_1, toxin passes to milk products at different levels. Therefore, it should be mentioned that reduced recovery may not mean reduced toxicity. Avoiding contamination appears to be the only practical and economical way to ensure safety of milk products for human consumption. Further studies are needed to understand the nature of the casein-AFM_1 association in order to completely explain the behavior of AFM_1 in dairy products.

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