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Research Article

Reduction of Aflatoxin M_1 Content during Manufacture and Storage of Egyptian Domaiti Cheese

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Abstract

Elevated levels of aflatoxin M_1 (AFM_1) in milk and milk products is considered to pose certain hygienic risks for human health. The maximum level of AFM_1 allowable in Egyptian milk is 50 ng/L and while a previous study found the majority of milk was below this level, some milk contained up to 250 ng/L. The aim was to determine what proportion of initial AFM_1 in milk is retained after manufacture into Domiati cheese and remains during 90 d storage. Milk was spiked with 1μg/kg AFM_1 then pasteurized at 63ºC for 30 min and made into Domiati cheese using salt additions of 6%, 8% and 10% (wt/wt). Cheese making was performed on 3 separate occasions. The AFM_1 levels in milk, cheese and whey were determined using an ELISA test kit. Pasteurization of milk caused ≤10% loss of AFM_1. About 60%, 58%, and 56% of total AFM_1 remained in cheese curd made using 6%, 8% and 10% salt respectively, with the residual being lost in the whey. After 2 wk storage at 20 ºC, all of the cheeses had a 17% reduction in AFM_1 compared to their levels after manufacture. With continued storage through 90 d the losses of AFM_1 were significantly different ($P < 0.05$) with reduction in AFM_1 or 20.5%, 21.4%, 22.0% for cheeses made using 6%, 8% and 10% salt respectively. Thus, including pasteurization of milk, conversion of milk into Domiati cheese and its subsequent storage period for 3 mo produced an overall 64% reduction of AFM_1. In conclusion, as well as avoiding contamination of milk with AFM_1 there is a lower health risk to the population from the presence of AFM_1 in milk when the milk is pasteurized and converted into Domiati cheese that is then stored for the customary 3 mo.

Keywords: Aflatoxin M_1, Domaiti Cheese, storage, ELISA

Introduction

Aflatoxins are generally produced in animal feeds by toxigenic fungi such as *Aspergillus flavus*, *Aspergillus parasiticus* and the rare *Aspergillus nomius* (Kamkar, et al 2011). They are both acutely and chronically toxic, mutagenic, teratogenic and carcinogenic compounds for animal and human (Deshpande, 2002; Ghazani, 2009; Maktabi et al., 2011 and Mohamadi Sani et al., 2012). Aflatoxin M_1 (AFM_1) is the principle hydroxylated metabolite of aflatoxin B_1 which is transformed at the hepatic level by

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means of cytochrome p450 enzymes and excreted into the milk in the mammary glands of both human and lactating animal after ingestion by the animal of pellets and forage contaminated with aflatoxin B_1 (Oveisi et al., 2007; Prandini et al., 2009; Hampikyan et al., 2010; Ayoub et al., 2011). It has been reported that there is a linear relationship between AFM_1 in milk and AFB_1 in the feed consumed by the animals with approximately 1% to 6% of the ingested AFB_1 appearing as AFM_1 in milk (Dragacci et al., 1995; Battacone et al., 2005; Fallah, 2010). Milk is a major food commodity for introducing aflatoxin into human diet and evidence of hazardous human exposure to AFM_1 through dairy products has been shown (Zinedine & Manes, 2009). Aflatoxin M_1 is resistant to thermal inactivation and not destroyed completely by pasteurization, autoclaving and other food processing procedures (Youssef & Marth, 1989; and Maktabi, & Fazlara, 2011).

Since the consumption of milk and milk products by human populations is quite high there is a risk of exposure to AFM_1 with infants and young children being at increased risk. Levels of AFM_1 in the diet are therefore important and AFM_1 in milk and dairy products should be controlled systematically to minimize such risk. Many countries have established regulations to control levels of AFB_1 in feeds and maximum permissible levels of AFM_1 in milk and cheese to reduce this risk (Sarimehmetoglu et al., 2004; Mahdiyeh et al., 2013). In the United States and Brazil (as well as in Codex) an action level for AFM_1 in fluid milk has been set at 500 ng/L (Shundo & Sabino, 2006; Codex Standard, 2008; Motawee et al., 2009) while the European Union (Commission Regulation, EC, 2006) has established a lower maximum allowable level for AFM_1 in milk of 50 ng/L and 250 ng/kg for cheese. Many other countries have followed the European Union standards (Dashti et al., 2009, Kamkar et al., 2011). In Egypt, the ministry of health established in 1990 that fluid milk and dairy products should be free from AFM_1 and currently the maximum permissible levels follow the European Union standard (Egyptian Standard, 2007).

So, for any country (including Egypt) any increase in the proportion of AFM_1 in milk and dairy products above the permissible limit of Codex and other countries can affect international trade of such milk products in global markets. Many studies have reported the occurrence of high levels of AFM_1 in milk and cheese in many countries that exceeded these maximum allowed limits (Cirilli & Cirilli, 1988; Oruc & Sonal, 2001; Motawee, 2003; Motawee et al., 2004, 2009; Tekinsen & Tekinsen, 2005; Yapar, et al., 2008; Atasever, et al., 2010; Tsakiris, et al., 2013).

Domiati cheese is the most popular soft white pickled cheese in Egypt (accounting for 75% of cheeses produced and consumed in Egypt). It differs chiefly from other pickled cheese varieties, such as feta, Brinza, or Telema cheese, in that the milk is salted (from 5% to 14%) before renneting depending on season and cheese ripening temperature (Abou-Donia, 1986; El-Baradei et al., 2007). Domiati cheese can be made from either cow or buffalo whole milk or their mixture. The salted milk can be curdled fresh of sometimes after pasteurization. No starter culture is added. It can be consumed fresh but more often after pickling in salted whey or a brine solution for up to 2 to 4 months (Zhang et al., 2003). The objective of the present study was to determine the effect of milk pasteurization followed by manufacture and pickling of Domiati cheese on AFM_1 levels in milk and cheese.

Materials and Methods

Cheese Making and Sampling

Domiati cheese was made (in duplicate) with some modifications according to Abou-Donia (1986) from 24 kg of cows milk (4% fat, 8% solids not fat). Milk was divided into two 12-kg batches that were spiked with 1.0 μg/kg of AFM_1 (Sigma chemical Co. Deisenhofen, Germany) and dissolved in milk, then pasteurized at 63°C for 30 minutes and cooled to 34±1°C. Then 0.8 g $CaCl_2$ was added to each batch to ensure good coagulation and curd formation and then each batch was divided into three 4-kg portions. To each portion of milk was then added 6%, 8% or 10%

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(wt./wt.) of NaCl, respectively. Then sufficient (1, 1.25 or 1.5 ml, respectively) standard strength calf rennet (Chr. Hansen's. Inc. Denmark) was added to produce a firm curd in 2 to 3 h at 34°C. The coagulum was then ladled out into 4-L steel molds lined with coarse cloth and the molds turned twice (after ~ 1 to 2 hour) and then allowed to stand 18 to 20 h to allow for whey drainage. The cheeses were removed from their molds and placed in small tin containers that were then filled with the respective cheese whey to exclude air and allow for pickling of the cheese. The containers were closed and stored at 20°C for 3 months.

Determination of AFM_1

Milk was sampled after spiking with AFM_1 and then after pasteurization. Cheese as sampled after curd formation, then before placing cheese in the pickling tins (day 0) and then every 15 d. Whey samples were collected at the same times. Each sample (milk, curd, whey, cheese) was stored at 4°C and analyzed for AFM_1 content within 12 h. Detection of AFM_1 was by an enzyme-linked immunoassay test kit (RIDASCREEN, R-Biopharm GmbH, Darmstadt, Germany) according to manufacturer's instructions (Anonymous, 1999) as briefly described below. The test kit included microtiter plates with immobilized AFM_1-antibody, AFM_1-enzyme conjugate, enzyme substrate (urea peroxide) chromogen (tetramethyl benzidine), and stop reagent 1M H_2SO_4. Other chemicals used included reagent grade methanol, n-heptane and dichloromethane (Merck, Darmstadt, Germany), and phosphate buffer saline at pH 7.2 prepared by mixing 0.55 g $NaH_2PO_4.H_2O$ with 2.85 g of $Na_2HPO_4.2H_2O$ and 9 g NaCl and then filled up to 1000 ml with distilled water. Pure AFM_1 from SIGMA (Deisenhofen, Germany) was used as a standard.

For milk and whey: Milk and whey samples (4 ml) were chilled to 4°C, centrifuged for 10 min at 3500 rpm, (Heraeus Megafuge 1.0, Thermo: Fisher Scientific, Inc., Waltham, MA, USA), and then the upper cream layer was completely removed by aspiration through a Pasteur pipette. The aqueous layer was then diluted 20 times (vol./vol.) with deionized water, then 100 µl placed into a microtiter plate sample well.

For cheese and curd: Curd and cheese samples (10 g) were coarsely ground and thoroughly mixed, without the addition of liquid using (Ultraturrax, IKA- Werke, Staufen, Germany) and then 2.000 $\pm$0.005g weighed into a centrifugal glass vial and 40 ml of dichloromethane was added and extracted by stirring/shaking the vial for 15 min. then the suspension was filtered and 10 ml of the extract was evaporated at 60°C under a weak nitrogen stream. The oily residue was redissolved in 0.5 ml methanol, 0.5 ml PBS buffer and 1 ml heptane and mixed thoroughly. After centrifugation for 15 min at 2700 g, the upper heptane-layer was completely removed. An aliquot of the lower methanolic-aqueous phase was carefully poured off using a Pasteur pipette. One hundred microliters of this aliquot was brought up to a 10% methanol content by addition of 400 µl Ridascreen buffer 1 and 100 µl was used per well in the test. In order to obtain sample AFM_1 concentration in ng/L, the concentration read from the calibration curve was further multiplied by a dilution factor 1 for milk and 10 for curd and cheese. Therefore, the mean detection limit for AFM_1 in milk and whey was 5 ng/L and in curd and cheese was 50 ng/kg.

ELISA test and standard curve procedure: AFM_1 standard solution was prepared containing (50, 100, 200, 400, and 800 ng AFM_1/L) for making a calibration curve. Samples (50 µl) in microtiter plate wells (in duplicate) were incubated for 60 min at room temperature in the dark, to allow antibody binding sites in the wells to be occupied proportionally to AFM_1 concentration. The liquid was then removed completely from the wells, which were washed twice with 250 µl of washing buffer and distilled water. In the next step, any remaining free binding sites were occupied by adding 100 µl of enzyme conjugate to the microtiter plate wells and incubated for another 60 min at room temperature (20 to 25°C) in the dark. Any unbound enzyme conjugate was then removed in a washing step. This was followed with 50µl of urea peroxide and 50 µl of tetramethylbenzidine and 30-min incubation at room temperature in the dark. , which then turned yellow on addition of 100 µl of the stop reagent. Yellow color was measured at 450 nm in ELISA reader (ELX-808, Winooski, VT, Inc., USA) against an air blank within 60 min, with AFM_1 concentration being inversely proportional to A_{450}.

Statistical Analysis

All the data were treated statistically using SAS (1996). To determine the effect of pasteurization, salting and storage treatments, a General Linear Model (GLM) was used with the equation: $Yij = \mu + \alpha j + \varepsilon ij$; where Yij is the AFM_1 level, μ the general mean, αj is the salting or storage effect, and εij is the residual error. The mean and standard error (SE) were used to express the results of the composition and AFM_1 levels.

Results and Discussion

Pasteurization of milk at 63 ºC for 30 min reduced AFM_1 content by ≤ 10% (Table 1). This is in agreement with El-Deeb et al., (1992) who reported a 9.5% drop in AFM_1. Mashaley et al., (1986) reported a 5.2 to 9.4% decrease in AFM_1 and AFM_2 in milk spiked with 5 and 10 µg/Kg and observed that the decrease during pasteurization was inversely proportional to the amount of toxin added. Similarly, Motawee & McMahon (2009) reported that pasteurization of milk caused ≤10% destruction of AFM_1 during Feta cheese making. Deveci (2007) investigated milk pasteurization at 72 ºC for 2 min and reported losses of AFM_1 of 12% and 9% in milk contaminated with 1.5 µg/Kg and 3.5 µg/L of AFM_1 respectively. An earlier study by Kiermeier & Mashaley, (1977) had reported 12% losses of AFM_1 in pasteurized milk at 75ºC for 40 sec. Thus, as shown through a number of studies, AFM_1 is relatively resistant to heat treatments such as pasteurization (Van Egmond et al., 1977; Wiseman & Marth 1983; Govaris et al., 2002; Oruc et al., 2006; Anfossi et al., 2012).

When this pasteurized milk was converted into Domiati cheese, there was a partitioning of AFM_1 between curd and whey that was dependent on the level of salt added to the milk. For cheese made from milks containing 6%, 8% or 10% salt, the levels of AFM_1 in the cheese curd was 2.73, 2.52 and 2.31 µg/kg, respectively. This increased concentration comes about as the casein and fat are concentrated as the cheese is made, while the level of AFM_1 in the corresponding whey was 0.36. 0.38 and 0.40 µg/kg, respectively. Thus there was a mean transfer of 60%, 58% and 56% of AFM_1 in the original milk into the cheese curd and 30%, 32% and 34% of AFM_1 was transferred into the whey with salt additions to milk of 6%, 8% and 10%, respectively. Our results are in agreement with other previous studies (Abd-Allalh, 1983; Yousef & Marth, 1989; Dragacci & Fremy, 1996; Motawee, 2003; Motawee & McMahon, 2009; Rubio et al., 2011).

Even though ~85% of the milk ends up as whey, a concentration of AFM_1 in cheese curd occurs because AFM_1 has an affinity to casein protein fraction in milk and it is more soluble in water than in oil. Levels of AFM_1 in Domiati cheese that are almost 3 times higher than in the milk from which the cheeses were made is in agreement with other studies on soft cheeses (Yousef & Marth 1989; Govaris et al., 2001; Prandini et al., 2009). Although, there have been other studies that have shown differing distributions of AFM_1 in milk between curd and whey. Some of these differences occur when there are different levels of AFM_1 in the milk. Some authors reported that half or more of the AFM_1 transfers into the whey: 50%, 61%, 66%, 86%, and 100% according to Stubblefield and Shannon, 1974; Wiseman and Marth, 1983; Blanco et al., 1988; Stoloff et al.,1981; and Purchase et al., 1972, respectively. In contrast, others have reported that most of AFM_1 transfers into the curd at levels of 66%, to 72%, 73% to 77%, 80%, and 100% according to Mashaley et al., 1986; El-Deeb et al., 1992; McKinney et al., 1973; and Blanco, et al., 1988, respectively. In a non-rennet cheese such as Ricotta cheese, most of the AFM_1 (~94%) goes into the hot whey when as the whey proteins precipitate (Cattaneo et al., 2013). These varying transfer rates can be ascribed to factors such type and degree of milk contamination, differences in milk quality, presence of curd fines in the whey, the cheese manufacture process, as well as experimental AFM_1 measurement techniques such as extraction method, methodology, and expression of the results.

When considered on a serving size (60 g of cheese compared to 250 ml of liquid milk) there is considerably less dietary exposure to AFM_1 when consuming Domiati cheese that has been pickled for 3 mo rather than milk. A serving of milk containing 500 ng/kg of AFM_1 (US allowable limit) would give an exposure of 125 ng AFM_1 while a serving of pickled Domiati cheese made from the same milk (pasteurized) would only give a exposure to ~45 ng AFM_1. Even consuming the cheese fresh after only 2 wk storage at 20 ºC, would still give a dose of <50 ng AFM_1 depending on the salting level used during cheese manufacture compared to their levels after manufacture. With continued storage through 90 d the losses of AFM_1 were significantly different ($P < 0.05$) with reduction in AFM_1. Continued slight transfer of AFM_1 from curd into whey during 90 days of storage is in agreement with others (Brackett and Marth, 1982b; Mashally et al., 1986; Fremy et al., 1990; Dragacci et al., 1995; Govaris et al., 2001; Motawee, 2003; and Motawee & McMahon, 2009). In contrast, the levels of AFM_1 during cheese ripening and storage of cheeses that are not stored in whey vary, as reported for Cheddar cheese (Brackett and Marth, 1982c), Brick and Limburger cheeses (Brackett et al 1982), Camembert and Tilsit cheeses (Kiermeir & Buchner, 1977) in which an increase in AFM_1 during the early stage of ripening was observed with decreases thereafter for cheeses prepared from naturally contaminated milk. While in Gouda (Van Egmond et al., 1977) or Mozzarella (Brackett & Marth, 1982b) cheeses there was no appreciable change during ripening for 6 or 4 months, respectively. These various results may be due to several factors such as the type of cheese, and as suggested (Brackett & Marth, 1982a) proteolysis during cheese ripening may release the toxin, or high lipolytic action during ripening of cheeses such as Teleme and release of free fatty acids may enhance release of AFM_1 from its hydrophobic bonds to casein (Brackett et al., 1982).

Since milk and dairy products are a source of many nutrients (especially protein and calcium) and consumed as a main food in many countries, the presence of AFM_1 is undesirable (Van Egmond., 1989; Mohamadi & Alizadeh, 2010 and Nilchian & Rahimi, 2012) and strategies for reducing dietary exposure to AFM_1 are important. For infants and young children, their exposure to contaminated milk and milk products puts them at high risk for ingestion of AFM_1 toxin (Karimi et al., 2007; Yapar et al., 2008; Sepehr et al., 2012; and Guo et al., 2013). For adults, consumption of dairy foods in the form of cheese, such as Domiati cheese, can help decrease risk of exposure to excessive levels of toxin.

Table (1) - Aflatoxin M_1 (AFM_1) content and concentration in milk spiked with 1.00 µg AFM_1 per kg and its partitioning between curd and its whey made using 4 kg of milk with 6%, 8% and 10% salt added to milk prior to manufacture of Domiati cheese.

Samples	**Sample Weight (kg)**	**Total AFM_1 (µg)**	**AFM_1 Concentration (µg/kg)**	**AFM_1 Recovery (%)**
Raw milk	4.00	3.96	0.99±0.003	99
Pasteurized milk	4.00	3.60	$0.90{\pm}0.001^{a}$	90
Salting 6%				
Cheese curd	0.88	2.40	$2.73{\pm}0.125^{a}$	60
Whey	3.34	1.20	0.36±0.027	30
Salting 8%				
Cheese curd	0.92	2.32	$2.52{\pm}0.082^{ab}$	58

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Samples	Sample Weight (kg)	Total AFM_1 (µg)	AFM_1 Concentration (µg/kg)	AFM_1 Recovery (%)
Whey	3.38	1.28	0.38±0.029	32
Salting 10%				
Cheese curd	0.97	2.24	2.31±0.037^{b}	56
Whey	3.41	1.36	0.40±0.029	34

Table (2) - Concentration of aflatoxin M_1 (AFM_1) in Domiati cheese made from 4 kg milk spiked with 1.00 µg AFM_1 per kg prior to pasteurization, and with salt additions to the milk of 6%, 8% and 10%, and subsequent during storage at 20°C in its whey, with percent loss of AFM_1 shown in parentheses.

Storage Time (days)	AFM_1 Concentration (µg/kg) (AFM_1 loss) (%)		
	6% Salting	**8% Salting**	**10% Salting**
0	2.73	2.52	2.31
15	2.268 ± 0.042^{a} (17.2%)	2.098 ±0.066^{abc} (17.0%)	1.928± 0.066^{cde} (16.9%)
30	2.180±0.021^{ab} (20.1%)	2.042 ±0.078^{bcd} (20.6%)	1.858 ± 0.059^{de} (19.9%)
45	2.178±0.066^{ab} (20.5%)	1.998±0.062^{bcde} (21.0%)	1.838 ± 0.061^{e} (20.8%)
60	2.185±0.062^{ab} (20.1%)	2.002±0.059^{bcde} (21.0%)	1.832 ± 0.067^{e} (21.2%)
75	2.178±0.061^{ab} (20.5%)	1.965 ±0.064^{cde} (21.4%)	1.808 ± 0.06^{e} (22.0%)
90	2.178±0.065^{ab} (20.5%)	1.988±0.066^{bcde} (21.4%)	1.812 ± 0.072^{e} (22.0%)

^{abcde} Means with same letter within all rows and columns were not significantly different, $\alpha = 0.05$

Conclusions

AFM_1 levels in milk, Domiati cheese and whey were determined using an ELISA test kit. Pasteurization of milk caused ≤10% loss of AFM_1. About 60%, 58%, and 56% of total AFM_1 remained in cheese curd made using 6%, 8% and 10% salt respectively after manufacture directly. When considered on a serving size (60 g of cheese compared to 250 ml of liquid milk) there is considerably less dietary exposure to AFM_1 when consuming Domiati cheese that has been pickled for 3 mo. rather than milk. A serving of milk containing 500 ng/kg of AFM_1 (US maximum allowance) would give an exposure of 125 ng AFM_1 while aserving of pickled Domiati cheee made from the same milk (pasteurized) would only give a exposure to ~45 ng AFM_1. Even consuming the cheese fresh after only 2 wk storage at 20 ºC, would still give a dose of <50 ng AFM_1 depending on the salting level used during cheese manufacture compared to their levels after manufacture. With continued storage through 90 d the losses of AFM_1 were significantly different (P < 0.05) with reduction in AFM_1.

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