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Detection of aflatoxins and novel simple regimes for their detoxification in milk and soft cheese

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ABSTRACT

The purpose of this study was to detect AFs in milk and in the most popular cheese samples produced that were sold in New Valley governorate, Egypt. Trials were also carried out to find a simple, available way to counteract AFs in these products. Sixty samples of marketable milk and soft cheese (locally manufactured) were randomly collected from El-kharja markets, New Valley governorate, Egypt. Aflatoxin (AFs) was detected quantitatively in the samples by using enzyme linked immunosorbent assay (ELISA) analysis. The positive results (tainted with AFs) indicated that 100% of the examined samples have level of Afs that exceed the allowable limit. Furthermore, locally manufactured soft cheese had higher AFs contamination than marketable milk. Additionally, the experimental trials to treat contaminated milk with microwave heating, Mish contaminated samples with lemon, and Kareish cheese with carbonated water revealed 9.4, 43.9, and 54.9% decline in Afs levels, respectively. It could be concluded that examined milk and milk products are frequently contaminated with AFs which exceed the allowable limits and every regimen that was employed to treat the contaminated specimen was helpful.

Introduction

Milk and milk products are abundant in essential elements, such as protein and calcium, which contribute to their widespread popularity (Omar, 2016). It could, nevertheless, be a source of harmful naturally occurring food pollutants (Iqbal *et al*., 2015). According to the Food and Agriculture Organization, approximately 25% of global food crops are tainted by mycotoxins (Omar, 2016). Eskola *et al*. (2019) reported that mycotoxins exceed European Union and Codex standards in over 25 percent of global agricultural output.

Aflatoxigenic fungi creates the most remarkable danger aflatoxins (AFs), primarily *Aspergillus flavus* and *A. parasiticus*, but sometimes occasionally *A. nomius, A. pseudotamarii* (Vaz *et al*., 2020), *A. parvisclerotegenus*, and *A. minisclerotigenes* (Pleadin *et al*., 2014). Aflatoxigenic fungi naturally produce these toxins as secondary metabolites, which act as pollutants during cultivation and/or food storage (Hooshfar *et al*., 2020). Tropic regions provide the ideal environment for the growth of aflatoxigenic fungi (Zebib *et al*., 2022).

Aflatoxin's chemical makeup mainly consists of difuran-coumarin atoms produced via the polyketide pathway. While 18 distinct forms of aflatoxins have been discovered, only six are widely acknowledged, known as B1, B2, G1, G2, M1, and M2 (Abbas, 2021).

Animal source food chains are contaminated with AFs when their fungi enter animal diets (Pleadin *et al*., 2014). Usually, *A. flavus* produces only B-type aflatoxins, while other *Aspergillus* species produce both B- and G-type aflatoxins (Zinedine and Manes, 2009). The presence of an oxygen atom in the cyclopentanone ring in G type is the only structural distinction from B type (FSSAI, 2016). Aflatoxin M1 and M2 represent the toxins B1 and B2 metabolized inside the animal body. The hydroxyl group's inclusion distinguishes M from B structure (Abbas, 2021).

Milk of animals whose feedstuffs have been polluted by AF-B1 and AF-B2 contain metabolites of M-type aflatoxins, which are typically not detected on crops (Iqbal *et al*., 2015) and are released in milk by the activity of cytochrome P450 in the liver of nursing animals (Jaiswal *et al*., 2018). Of the AFB1 consumed, between 0.3 and 6.2% is transformed into the monohydroxy metabolite aflatoxin M1 (Vaz *et al*., 2020). This transmission rate depends on animal genetics, seasonal variation, milking technique, and environmental conditions (Iqbal *et al*., 2015).

The presence of mycotoxins, specifically aflatoxins, in food, agricultural goods, and feed has emerged as a pressing health concern due to their potential to induce poisoning, severe health implications for both animals and humans and substantial economic ramifications (Mukhtar *et al*., 2023). Cattle that consume feed tainted with AFB1 have a risk of developing health problems since it affects immune system function, decrease milk production, damages liver function, and makes them more susceptible to illness (Sabatelli *et al*., 2023).

The genotoxic, cytotoxic, and carcinogenic effects of AFM1 are evident, albeit 10% less damaging than AFB1 (Ismaiel *et al*., 2020). AFM1's possible effects on human health have been highlighted by the International Organization of Research on Cancer (IARC, 2015), which has classified it as one of the " carcinogens type 1" with AFB1. Owing to aflatoxins' extreme toxicity, numerous nations have created stringent laws governing their presence in food, particularly milk (Suresh *et al*., 2021).

Limits on mycotoxin levels in food and feed have already been established in more than 100 nations. However, there are unclear restrictions in other nations, particularly developing nations (Matabaro *et al*., 2017). The upper limit of AFM1 in milk and dairy goods is 50 ng/kg, according to regulations published by the European Commission (2006) and Codex Alimentarius Commissions (2001). Conversely, those are subject to US laws that set a limit of 500 ng/kg AFM1 (Omar, 2016). Liquid milk and milk-related goods must not include AFM1, according to Egyptian Minister of Health rules (Egyptian standards, 2003).

Pasteurization or milk processing into cheese does not affect AFM1, which remains reasonably constant in fresh and manufactured milk products (Sarımehmetoglu *et al*., 2004). Many strategies have, therefore, been developed to render aflatoxins inactive (Suresh *et al*., 2021). Several methods exist for lowering mycotoxigenic fungi's growth and mycotoxin production in food (Mukhtar *et al*., 2023). Numerous physical, chemical,

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and biological techniques for reducing aflatoxins have been described during the past few decades (Zhang *et al*., 2021). For the detoxification process to be effective, the procedure needs to be low-cost, leave no toxic residues, and have little effect on the item's quality (Zhang *et al*., 2021).

The majority of these techniques, however, are not always valid since they could be costly or energy-intensive and even result in a loss of quality (Suzuki *et al*., 2002). As a result, scientists have recently become increasingly interested in developing a safe, efficient, and energy-saving technique to detoxify aflatoxins (Zhang *et al*., 2012). However, there is still a great need to combat mycotoxins and other fungal-related problems in society through a worldwide strategy (Mukhtar *et al*., 2023).

Consumers in developed nations, specifically those in rural regions, encounter food safety and security difficulties due to their dependence on domestic foods (Marroquín-Cardona *et al*., 2014). Aflatoxin M1 contamination of dairy products and milk is a significant issue, especially in developing countries (Iqbal *et al*., 2015).

This investigation aimed to ascertain whether AFs were present in milk, and the most common cheese samples made and sold in the New Valley governorate, and to estimate the concentration of AFs. Additionally, trials were conducted to discover an easy, available, novel method to combat AFs in these products.

Materials and methods

Samples collection

Sixty samples of marketable milk, Kareish, and Mish cheese products (20 samples each) were randomly collected from El-Kharja markets during April to May 2023. The collected samples were promptly transported to the laboratory packed in ice packs and subsequently stored at a temperature of -20°C until analysis. All samples underwent aseptic processing for downstream analysis using sterilized components.

Analyses of samples

Sixty samples of milk, Kareish, and Mish were analyzed by using commercial ELISA kits (Human total aflatoxins ELISA kits, CO. of Glory Science, LTD, China). Out of them, thirty samples that were positive for aflatoxin in milk, Kareish, and Mish (10 samples each) were subjected to three different treatments before re-analysis as follows;

Milk samples: about 25 ml were treated by exposure to microwave heating at a medium power level (Kenwood; 800 W power, 20 L, MWM100, China. For 5 minutes until boiling according to Smajlović`*et al*. (2012).

Kareish cheese samples: about 50 g were mixed and then soaked in 100 ml carbonated water (natural mineral water) containing the following minerals in mg/L: 290 HCO3, 48 Ca, 29 Mg, 8.9 NO3, 5.6 Na, 3.0 SO4, 3Cl, 0.9 K, and 0.1F; with a pH of 5.50 (from SAN BENEDETTO, Italy) for at least one hour, as described by Samiye *et al*. (2024) and Zhang *et al*. (2012) with some modification.

Mish cheese samples: about 50 g were mixed with lemon juice (20 ml) for at least half an hour (Hossein *et al*., 2017; Gammariello *et al*., 2021).

AFs separation protocols for ELISA technique

Milk

Milk was used immediately for the analysis after centrifugation for 10-20 min at a speed of 3500 rpm. For analysis, the lowest layer was utilized (Al-Mossawei *et al*., 2016).

Cheese

Four ml of 100% methanol was added to 1 g of minced cheese samples. Violently vortexed by hand for five minutes. The samples underwent centrifugation for 10 minutes at a rotational speed of 4000 revolutions per minute, one ml of the effluent was transfered into a separate tube and completely desiccated by employing a rotatory evaporator operating at a temperature of 70ºC. Each dehydrated sample was treated with 800 µl of 1x PBS and subjected to vortexing for one minute (AL-Mossawei *et al*., 2016).

Assay for the quantitative detection of AFs

Human total aflatoxins commercial ELISA kits (CO. of Glory Science, LTD, China) was used to detect total aflatoxins in the prepared milk and cheese samples at 450nm. The experiment was conducted following the manufacturer's guidelines and by using duplicates of each standards and samples.

Statistical analysis

To distinguish substantially different means, one-way ANOVA and multiple comparison tests were utilized; significance was determined at the probability of $p < 0.05$. Statistical Product Service Solution (SPSS) version 21 was used for all the analyses. The data was also summarized using Microsoft Excel and qualitative statistics.

Results

Results in Table 1 showed that 100% examined samples were positive for Afs. Significant aflatoxin levels between milk (3.84±0.1 ppb), Kareish (14.99±2.4 ppb), and Mish (20.0±3.2 ppb) were detected. Moreover, the effect of different treatments on AFs-contaminated specimens is shown in Table 2. The total aflatoxin level in milk was 3.60±0.17 ppb before treatment and 3.27±0.05 ppb after microwave treatment; the percentage of aflatoxin decrease was 9.4% in the same samples. The total aflatoxin level of Kareish cheese was 20.49±2.8 ppb before treatment and 9.25±2.2 ppb after carbonated water treatment, with 54.9% reduction percentage of aflatoxin in the same samples. The total aflatoxin level of Mish cheese was 21.31±3.4 ppb before treatment and 11.96±1.9 ppb after lemon juice treatment, with 43.9% decline percentage of aflatoxin in the same samples.

Table 1. Detection of AFs (ppb) in milk and cheese samples without treatment using ELISA technique.

Type of Sample	No.	AFs Mean±SE	Min.	Max.
Milk	20	3.84±0.1^{c}	2.94	4.56
Kareish	20	14.99±2.4^{a}	3.47	30.46
Mish	20	20.0±3.2^{b}	3.77	40.5

Values followed with different superscript letters ($^{a, b, c}$) in the same column are significant at $p \leq 0.05$

Discussion

In Egypt, marketable milk and the kinds of cheeses studied in this paper are trendy. The presence of AFs reduces agricultural product quantity and quality, whereas efficient prevention of AF contamination has the opposite impact (Udomkun *et al*., 2017).

Aflatoxin in the examined marketable milk and cheese products samples were 100% in different levels exceeding the permissible level of AFM1 in milk (Egyptian standards, 2003), similar findings were recored by El-Hofi and Abo El-Naga (2021) who reported that AFM1 level in milk and cheese were higher than those allowed by Egyptian law. However, in El-Hofi and Abo El-Naga's study, they found negative aflatoxin specimens contrary to ours, this may be attributed to the variations in the type of cheese, toxin analysis techniques, cheesemaking processes, and milk contamination levels (Mohammadi *et al*., 2022). The obtained results agree with Silva *et al*. (2023) who detected AFM1 in 100% of cheese samples, which ranged from 0.026 to 0.132 µg/kg; however, none of the cheese samples were higher than the acceptable level of the European Union.

Table 2. Detection of AFs (ppb) before and after treatment of milk and cheese samples using ELISA technique.

Sample kind	No. of treated samples	Treatment		AFs Mean±SE	Min.	Max.	Percentage of decline
Milk	10	Microwave	BF	3.60±0.17	3	4.05	9.40%
			AF	3.27±0.05	3.13	3.42	
Kareish	10	Carbonated water	BF	20.49±2.8	14.05	30.46	54.90%
			AF	9.25±2.2	3.81	15.83	
Mish	10	Lemon juice	BF	21.31±3.4	15	39.84	43.90%
			AF	11.96±1.9	3.22	17.39	

Aiad and Abo el-makarem (2013) assessed the frequency of AFM1 in milk and cheese products in Alexandria, found all positive samples of raw milk, and cheeses, surpass Egyptian regulations.

Previous studies have shown that Aflatoxin B1 and B2 in food items consumed by ruminants are broken down and released as aflatoxin M1 and M2 in milk (Ferrari *et al*., 2023). The present investigation estimated the total aflatoxins in milk and dairy products regardless their types.

According to the current study, Mesh cheese contained the highest AFs concentration, followed by Kareish cheese and finally milk specimens, which might be because AFM; the main aflatoxin in milk and its products, which binds to milk proteins, especially casein (Pietri *et al*., 2016), which is nearly concentrated in cheese (Mozaffari *et al*., 2020). Straight with that, separating the cream will eventually raise the AFM1 content in dairy goods by four times since AFM1 is unevenly distributed in milk and is more closely associated with the protein component of milk (casein) (Picinin *et al*. 2013; Škrbić *et al*. 2015).

Horizontal with a study by Amer and Ibrahim (2010) who evaluated the level of AFM1 in varieties of Egyptian-produced cheese, it was reported that samples of soft and hard cheese had higher AFM1 concentrations than samples of raw milk and cheese that had been processed. Various factors, including manufacturing processes, variations in milk contamination, cheese type, ripening circumstances, and testing methodologies used, have been attributed to these uneven results (Sarımehmetoglu *et al*., 2004).

Studies have shown that the content of AFM1 in different soft cheeses is around three times higher and approximately five times higher in hard cheeses, more than in the milk used to make the cheese itself (Mozaffari *et al*., 2020). That is clarified in Kareish chesse, which has no additives. Additionally, while other type of cheese, adding flavors is possible based on its type. Herbs, spices, and sweet and fiery peppers are a few typical ingredients (Boor, 2005), as they have been added during Mish manufacturing and might be the causative of its highest AFs concentration as they are considered another source of other types of aflatoxins in Mesh samples in addition to AFM1.

Treatment of milk with microwave is consider a new trial to combat AF-contaminated milk. The results revealed a 9.4% decline in AF in milk samples after treatment. There have been few studies on the subject, and the results are conjectured on using microwave heating to destroy aflatoxins from various food grains (Suresh *et al*., 2021). The results of the current study were parallel with the previous research that told when the aflatoxin-contaminated corn microwave cooked for 5.5 min at a power output of 1650 W, Aflatoxin B1 and B2 were effectively reduced by the percentage of 36% and 58%, each (P'erez-Flores *et al*., 2011). In addition, according to Mobeen *et al*. (2011), microwave heating of groundnuts and products derived from them decreased the amount of aflatoxin B1 to the percentage of 50–60 and decreased until aflatoxin B2 was undetectable. However, it is not in the same decline percentage as ours, which may be attributed to the difference in aflatoxins samples (corn and milk). AFB1 degradation in these studies mainly depended on the high temperature generated by microwave heating; an effective decrease of AFB1 needed a temperature of 130–150◦C or above. However, when it came to reducing AFB1 in corn, depending on high temperatures, it had some significant downsides. For example, microwaves that heat up quickly induce heat-damaged corn kernels and crack more frequently. This is because microwaves generate volumetric heat, which can cause textural damage. After all, it is difficult to manage the ultimate product temperature (Pankaj *et al*., 2018). Some earlier papers found that the presence of water molecules helps destroy aflatoxins by microwave. These results support our sound idea of heating contaminated milk with a microwave. The lactone ring was opened to create a terminal carboxylic acid (Kabak, 2009), and AFB1's breakdown temperature decreased (Samarajeewa *et al*., 1990). According to Hu (2016), AFB1 typical substance in water could be broken down by microwave heating at 80◦C, but edible oils required a temperature higher than 200◦C to break down AFB1 (Samarajeewa *et al*., 1990).To lower AFB1 in maize, it could be helpful to do studies using water as a microwave heating medium. In the literature, some researchers mentioned that the combination of water and microwaves had excellent potential as a postharvest disinfestation treatment (Soto-Reyes *et al*., 2017).

The results of this paper on Kareish cheese after novel treatment with carbonated water, AFs were reduced by 54.9%. Recent studies have demonstrated that electrolyzed oxidized water (EOW), produced by electrolyzing electrolyte solution, possesses highly effective antibacterial action (Abadias *et al*., 2008). The process of electrolyzing water containing 1% sodium chloride (NaCl) yields EOW, also known as electro-activated water (Pandey, 2022). For the purpose of sanitizing surfaces and guaranteeing food safety, electrolyzed water (EW) had been used as a disinfectant and cleaning agent, particularly in the food sector (Samiye *et al*., 2024). The obtained results flattened with Zhang *et al*. (2012), who reported applying an additional novel technique called acidic electrolyzed oxidizing water to inhibit AF pollution. This technique involves preparing an electrolyte solution using an ion-exchange membrane electrolysis apparatus to clean AF-B1 from naturally contaminated groundnut samples. After soaking in the solution, the amount of AF-B1 in the groundnuts dropped by around 85%. Amazingly, the groundnuts' color and nutritional makeup did not significantly change after treatment.

Moreover, the work levels of AFs in Mish specimens after treatment with lemon were reduced by 43.9%. Organic acids have been routinely used in the food business to degrade aflatoxin (Pandey, 2022). Citric acid's impact on the degradation of AF-B1 and AF-B2 in extruded sorghum illustrates this phenomenon well (Jalili and Jinap, 2012). Tartaric acid had the most significant AF-B1 reduction rate, followed by citric, lactic, and succinic acid, in that order (Udomkun *et al*., 2017). After these acid treatments, AF-B1 changes into b-keto acid, which then changes into AF-D1, less harmful than AF-B1 (Mendez-Albores *et al*., 2005). According to Lee *et al*. (2015), after being immersed in 1.0 N tartaric acid, lactic acid, and citric acid for eighteen hours at room temperature, AFB1-contaminated soya bean reduced its level by 95.1, 92.7, and 94.1%, respectively. This differs from our study in immersion for a long time, using pure citric acid, and subsequently differs in the declining percentage of aflatoxins. Also, the results agree with Rastegar *et al*. (2017) who reported that roast pistachio nuts acidulated with lemon juice for 60 minutes at 120°C, resulted in reduction in AFB1 level by 50.2%. The results were similar to Jalili's (2015) work; when food and feed were treated with higher concentrations of citric acid and other compounds, there was a documented drop in AFs. 1N aqueous citric acid was used to eliminate aflatoxin (AFB1 and AFB2) in maize grain by 96.7% (Hwang and Lee, 2006). A comparable investigation found that employing 1N aqueous citric acid reduced commercial AFB1-contaminated feed by 86% (Mendez-Albores *et al*., 2007). The results confirmed by Mendez-Albores *et al*. (2008) When exposed to alkaline and acidic conditions; as a result, AFs can have opened lactone rings, transforming the AFs into a water-soluble molecule called beta-keto acid that is readily washed out of the sample. Furthermore, beta-keto acid can be hydrolyzed to produce AFD1, a non-fluorescent molecule with phenolic characteristics and no lactone group (obtained from the decarboxylation of AFB1's lactone ring-opened derivative). The study of Gammariello *et al*. (2021) on cheese products, using the lemon and chitosan separately had the best results as an antimicrobial activity than when the two compounds Extract were mixed.

Conclusion

The current investigation showed that milk and milk products frequently contain AFs contamination which exceed the allowable limits. Since AFM is a severe danger for dairy products, and food safety, healthcare authorities should implement strict guidelines and standards, including inspection and monitoring of dairy products and feedstuffs for aflatoxins. Further, all used regimes for treating the contaminated specimen are effective. Carbonated water was the first new area in the literature to study its effect on AF degradation. Owing to lack of research studies on the effect of the applied treatment on milk products. Further investigation is required to comprehend the ways in which each of these

management affect.

Conflict of interest

The authors declare that they have no financial or personal relationship (s) that may have inappropriately influenced them in writing this article.

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