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Research article

Occurrence of aflatoxin M1 in cheese products commonly available in Sri Lankan market

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A R T I C L E I N F O

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A B S T R A C T

Aflatoxins (AFs) are a group of mycotoxins produced by certain fungi of *Aspergillus* spp. AFs of major concern are B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), and M1 (AFM1). AFM1 is a hydroxylated metabolite of AFB1 formed inside the animal's body which is excreted into milk of cows that consumed AFB1 contaminated feed. Consumption of AFM1-contaminated milk and subsequent dairy products causes negative health effects in consumers. This study was conducted to determine the occurrence and levels of AFM1 in cheese products available in the Sri Lankan market where AFM1 is not regularly monitored in milk while having an outdated regulatory limit of 1 ppb established for dairy products. Processed cheese (n = 28), hard cheese (n = 14), semi-hard cheese (n = 5), and soft cheese (n = 3) representing seven popular brands were collected. The samples were analyzed by Ultra High-Performance Liquid Chromatography Fluorescence Detection. AFM1 was detected in 40 samples (80 %), while 17 (34 %) and 37 (74 %) of the samples had AFM1 levels exceeding the maximum permitted limit set by Codex Alimentarius Commission (0.5 ppb) and the Netherlands (0.2 ppb). Further, 10 samples violated the Sri Lankan maximum limit of 1 ppb. Thirteen out of the 14 hard cheese (92.9 %, 0.11–14.43 ppb) and all semi-hard cheese samples (100 %, 0.29–0.65 ppb) contained AFM1. Most of the soft (66.7 %, 0.35–0.45 ppb) and processed (71.4 %, 0.11–1.35 ppb) cheese samples had AFM1. Most of the locally manufactured cheese products in Sri Lankan market may pose health risks to consumers. The results highlight the significance of regular monitoring of AFM1 in dairy products and the importance of updating regulations on par with international standards concurrently to ensure consumer safety.

1. Introduction

Aflatoxins (AFs) are a group of mycotoxins of public health concern that are produced by fungi of *Aspergillus* spp. AFs have carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive effects [1–3]. The naturally occurring AFs exist in four varieties: B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2) [2] and intake of AFs-contaminated feeds can affect the health and

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productivity of animals [4]. The formation of AFM1, a hydroxylated metabolite of AFB1, occurs in the liver and it is excreted into milk in the mammary gland of dairy cows fed with AFB1-contaminated feedstuffs [5]. The conversion factor of AFB1 from animal feed to AFM1 in raw milk is 0.30–6.20 %, depending on the genetics, lactation stage, milk production, and other cow factors [5]. AFM1 can be found in cow's milk 12–24 h after the first intake of AFB1 [6,7]. Consumption of AF-contaminated dairy products can have negative health effects on consumers [8]. One of the most popular dairy products is cheese, which is produced using a variety of methods and categorized according to its varieties [9,10] and published data available on the AFM1 contamination in cheese in the South Asian region is very scarce.

The occurrence of AFs in cheese could be from three sources. i.e. direct contamination of cattle feed with AFB1 and biotransformation into AFM1 in milk [11,12], presence of AFM1 in other dairy additives [11,13] and contamination of mycotoxin producing spoilage/technological filamentous fungi exogenically or endogenically [14]. The cytotoxicity, genotoxicity, and carcinogenicity of AFM1 are well-established [15]. Therefore, this toxin, was earlier classified as a Group 2B human carcinogen [16] and in 2002, it was upgraded to Group 1 human carcinogen [15].

AFM1 is relatively stable during pasteurization, ultra-high temperature, storage, and processing [17,18]. Thus, the consumption of heat-treated milk may contain AFM1 which could be transferred to humans. Therefore, the determination of AFM1 in milk and dairy products is essential for regulatory purposes which require suitable analysis techniques like enzyme-linked immunosorbent assay, high-performance liquid chromatography (HPLC), and HPLC coupled tandem mass spectrometry.

The CODEX Alimentarius Commission has established a maximum limit of 0.50 μg/kg for AFM1 in milk and dairy products [19]. The maximum limit(s) for AFM1 set by the European countries for cheeses are different (e.g. Netherlands 0.20 μg/kg, Austria, Switzerland and France 0.25 μg/kg; Italy 0.25 μg/kg for soft cheeses and 0.45 μg/kg for hard cheeses μg/kg) [20]. The European Commission has specified a maximum level of AFM1 for milk used for milk products as 0.05 μg/kg [21]. Further, part 1 in section 1 of Gazette Extraordinary of the Democratic Socialist Republic of Sri Lanka issued on 1993.10.14 has enforced a regulatory limit of 1 μg/kg for AFs in milk and/or milk products intended for human consumption.

It could be observed that developed countries have defined their own maximum limits of AFM1 in cheese which are generally low (e.g. 0.20–0.25 μg/kg) or opted for CODEX regulations, while developing countries lack such regulations or have established higher regulatory limits for AFM1 in cheese (1.00 μg/kg). According to Liu, Y., & Wu, F., it has been found that developing countries suffer from higher occurrences of hepatocellular carcinoma (HCC) caused by aflatoxins [22]. Therefore, it is crucial to regulate AFM1 exposure in humans, by means such as cheese and other dairy products, especially in developing countries, as some of these countries are bound by outdated regulations. Therefore, this study was aimed at determining the occurrence and levels of AFM1 in commercially available cheese products marketed in Sri Lanka to ensure consumer safety.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used were HPLC grade. Methanol (99.7 %) and glacial acetic acid (100 %) were purchased from Sigma-Aldrich, USA while acetonitrile (99.8 %) was purchased from Merck Life Sciences Pvt Ltd, Mumbai, India. Technical grade trifluoroacetic acid (99 %) and sodium chloride were purchased from VWR International, Belgium. The standard solutions of AFB1 (2000 μg/L), AFB2 (500 μg/L), AFG1 (2000 μg/L), AFG2 (500 μg/L), and AFM1 (500 μg/L) were purchased from Fermentek LTD., Jerusalem, Israel. All the standards were stored at −20 °C in the dark. Ultrapure (Grade I) water used in the experiment was obtained from EX-1101 Crystal EX HPLC Water Purification System (Adrona, Latvia).

2.2. Sample collection

Commonly available processed cheese (n = 28), hard cheese (n = 14), semi-hard cheese (n = 5), and soft cheese (n = 3) representing seven brands were collected from the local market in Sri Lanka using a stratified random sampling method. Whenever available a minimum of three batches from each cheese type were collected.

2.3. Extraction of AFs

The ground/homogenized cheese samples were accurately weighed 25.00 ± 0.05 g into 50 mL clean centrifuge tubes. Then 2.50 ± 0.05 g NaCl and 100 mL of methanol:water (80:20 v/v) were added and blended at high speed (~10800 RCF) for 1 min. The samples were centrifuged (Eppendorf Centrifuge 5810 R – Benchtop centrifuge, Germany) at ~1250 RCF for 10 min at room temperature. The supernatant was passed through the fluted filter paper (VICAM, USA). Then 10 mL from the filtrate was transferred into a clean 50 mL centrifuge tube and 40 mL of water was added, vortexed, and filtered through a 1.5 μm microfiber filter (VICAM, USA). Then 20 mL of the diluted filtrate was passed through an immunoaffinity cleanup column (AflaTest®, VICAM, USA) at a rate of 1 drop/sec until air came out. Next, 2 × 10 mL water (1–2 drops/sec) was passed through the immunoaffinity cleanup column until air came out. The bound AFs were eluted into a 2 mL microcentrifuge tube with 1 mL acetonitrile at a rate of 1 drop/sec.

2.4. UHPLC analysis

AFM1 was detected using UHPLC-FLD system (Shimadzu Nexera X2, Kyoto, Japan) consisting of a degassing unit, communication

Table 1
Back calculated concentration of calibration curve standards for AFM1.

	Nominal concentration (μg/kg)					Slope	Intercept	r^2
	0.33	0.56	1.05	2.84	4.90	–	–	–
Day 1	0.34	0.42	1.07	3.05	5.02	12192.70	−1906.51	0.9985
Day 2	0.39	0.58	1.13	3.02	4.76	15165.70	−3987.15	0.9991
Day 3	0.31	0.61	0.85	2.68	4.33	7441.96	−184.79	0.9996
Mean	0.35	0.53	1.02	2.92	4.70	–	–	–
SD (±)	0.04	0.10	0.15	0.21	0.35	–	–	–
CV (%)	12.53	18.70	14.34	7.08	7.50	–	–	–
% Nominal	94.26	105.07	103.35	97.25	104.20	–	–	–

Table 2
Performance characteristics of the analytical method for determination of AFM1 in cheese.

AFM1 Spiked Level	Mean Recovery (%)	RSDr (%)	$RSDw_R$ (%)	LOD (μg/kg)	LOQ (μg/kg)	Uncertainty (%)
0.25 μg/kg	79 %	20 %	20 %	0.042	0.126	9.0 %
0.50 μg/kg	87 %	18 %	19 %			9.0 %
1.00 μg/kg	79 %	16 %	19 %			8.8 %

Repeatability relative standard deviation (RSDr); within-laboratory reproducibility relative standard deviation ($RSDw_R$); LOD - limit of detection; LOQ - limit of quantification.

Table 3
Presence of Aflatoxin M1 in different cheese types in the local market in Sri Lanka.

Cheese type	Brand	AFM1 positive samples			AFM1 detected range (μg/kg)	Mean of AFM1 levels of positive samples (μg/kg)
		>LOD (0.02 μg/kg)	> Dutch (0.20 μg/kg)	>CODEX (0.50 μg/kg)		
Processed	A	10/10 (100 %)	10/10 (100 %)	4/10 (40 %)	0.21–1.35	0.55^a
Processed	B	8/9 (89 %)	6/9 (67 %)	1/9 (11 %)	0.11–0.92	0.32^b
Processed	C	0/6 (0 %)	0/6 (0 %)	0/6 (0 %)	ND	
Processed	E	0/1 (0 %)	0/1 (0 %)	0/1 (0 %)	ND	
Processed	F	2/2 (100 %)	2/2 (100 %)	0/2 (0 %)	0.28–0.35	0.32^b
Hard	A	3/3 (100 %)	2/3 (67 %)	1/3 (33 %)	0.11–0.64	0.28^c
Hard	D	8/8 (100 %)	8/8 (100 %)	8/8 (100 %)	1.20–14.43	4.47^d
Hard	G	2/3 (67 %)	2/3 (67 %)	1/3 (33 %)	0.39–0.63	0.34^c
Semi-hard	A	5/5 (100 %)	5/5 (100 %)	2/5 (40 %)	0.29–0.65	
Soft	B	2/3 (67 %)	2/3 (67 %)	0/3 (0 %)	0.35–0.45	

Significantly ($p < 0.05$) different AFM1 means are denoted by different letters.

bus module, pumps (LC-30AD), autosampler (SIL-30AC), column oven (CTO-20AC) and a fluorescence detector [RF-20A (excitation 360 nm, emission 450 nm)] with a reversed-phase C18 column (4.6 × 250 mm, particle size 5 μm, Phenomemex Inc., Torrence, CA, USA). The mobile phase consisted of water, acetonitrile, and methanol (60:24:16 v/v/v) at a flow rate of 1 mL/min, and 10 μL of the sample was injected. The AFM1 in cheese samples was quantified using an external standard calibration curve. The average Limit of detection (LOD) and Limit of quantification (LOQ) were calculated based on the signal-to-noise ratio of 3:1 and 9:1, respectively. The performance characteristics of the analytical method were verified using 20 blank samples and 20 spiked samples at each 0.25, 0.50 and 1.00 μg/kg levels following the guidelines provided in Commission Implementing Regulation (EU) 2023/2782. These blank and spiked samples represented soft, processed, semi hard, and hard cheeses. The repeatability (RSDr), intermediate precision (RSDi) and % uncertainty were calculated for each spike level separately. The linearity of the calibration curve was established by back calculating the concentration of calibration curve standards for AFM1 (Table 1). This method is accredited under ISO 17025 by the Sri Lanka Accreditation Board. Furthermore, laboratory proficiency tests by Progetto Trieste, Italy are conducted using this method.

2.5. Statistical analysis

Each positive sample was analyzed for a second time for AFM1 and the average values were reported. The mean AFM1 concentrations among different cheese types and brands were compared using one-way ANOVA (p vlaue≤0.05) and Tukey's test was carried out for pairwise comparisons using Minitab 18 (Minitab, LLC).

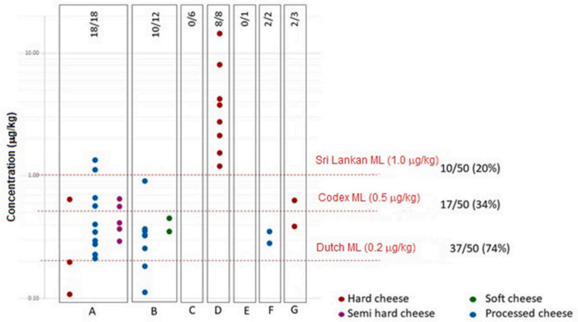

Fig. 1. Occurrence of AFM1 in different cheese types available in Sri Lanka.

3. Results and discussion

3.1. Validation

The performance characteristics of the analytical method for determination of AFM1 in cheese is presented in Table 2. The average recovery of the method for different spike levels were within the required 70–120 % range. Further, the repeatability relative standard deviation (RSDr) and within-laboratory reproducibility relative standard deviation ($RSDw_R$) were below 20 % as required by Commission Implementing Regulation (EU) 2023/2782. The linearity of the calibration curve was over 0.998 (Table 1). The laboratory participated in the proficiency testing scheme offered by Progetto Trieste in March 2022 on determining AFM1 in hard cheese (assigned value: 0.371 μg/kg) and fresh cheese (assigned value: 0.239 μg/kg) and obtained satisfactory Z-scores of 0.21 and 0.41, respectively.

3.2. AFM1 in cheese products

The presence of AFM1 in cheese raises significant concerns regarding the safety of the products. In this study, AFM1 was detected in 40 samples (80 %) out of the 50 samples analyzed. The occurrence and levels of AFM1 in cheese samples analyzed were summarized in Table 3. Seventeen (34 %) and 37 (74 %) samples exceeded the regulatory levels for AFM1 set by Codex Alimentarius Commission (0.50 μg/kg) and the lowest level in the Europe (Netherlands- 0.20 μg/kg), respectively in which the AFM1 ranged between 0.26 and 14.43 μg/kg (Fig. 1). Further, 10 samples (20 %) violated the Sri Lankan maximum limit of 1 μg/kg and ranged from 1.13 to 14.43 μg/kg which includes all hard cheeses of brand D and two processed cheeses of brand A (Fig. 1).

Thirteen out of the 14 hard cheese (93 %; 0.11–14.43 μg/kg) and all semi-hard cheese samples (100 %; 0.29–0.65 μg/kg) contained AFM1. Further, most of the soft (67 %; 0.35–0.45 μg/kg) and processed (71 %; 0.11–1.35 μg/kg) cheese samples had AFM1. The amount of AFM1 contamination was higher in high milk-containing products. AFM1 levels of hard cheese samples were significantly ($p < 0.05$) different from AFM1 levels in processed, semi-hard, and soft cheese samples. Several studies have shown that about 30 % of AFM1 have an affinity to bind with milk non-fat solids which contain proteins, mainly casein due to the semi-polar characteristic of AFM1 [23,24] and concentrated in curd after draining [25]. The hard cheese products which have more milk casein and low water content had higher AFM1 levels compared to other cheese types. AFM1 in all the hard cheese samples of brand D (1.20–14.43 μg/kg) was above both Dutch and Codex regulatory limits, and the highest AFM1 contamination was also reported in brand D (14.43 μg/kg). However, hard cheeses of brands A and G contained lower AFM1 (brand A; 0.11–0.64 μg/kg and brand G; 0.39–0.63 μg/kg respectively) compared to brand D. Risk factors such as climatic conditions (mainly humidity) of the farm, feeding practices, carry-over rate of AFM1, and milk collection practices (whether from a single farm or a collection network) contribute to the AFM1 contamination in milk used for cheese production [26]. According to our results, the highest risk for AFM1 contamination was posed by manufacturer/brand D located in the hill country of Sri Lanka, where the humidity is high and the entire milk production is from a single farm managed intensively with the feeding of high amount of concentrates to high yielding animals. This could be supported by a previous study, where a high level of AFM1 contamination in milk was reported from the same manufacturer [27]. Cow milk coming from a wider collection network is used for the production of cheese brands A and B. We have observed that milk coming from a larger network generally has lower AFM1 levels compared to large-scale single farms probably due to some level of dilution of AFM1. This dilution is possible as certain farmers in milk collection networks extensively manage their cattle with little or no concentrate feeding.

The study observed a significant difference in AFM1 contamination between locally manufactured (brands A, B, D, F, and G) and imported (brands C and E) cheese. In developed countries, milk is obtained from free-grazing cows or cows fed with highly nutritious pasture [28,29]. In addition, stringent quality control measures have been implemented for animal feed, significantly reducing the likelihood of aflatoxin contamination [30,31]. In contrast, in developing tropical countries the pasture provided to the animals is low in nutrition [32–34]. Hence dairy cows are often fed with concentrates that are primarily composed of maize, compound feed, and cocont poonac which are more prone to AF contamination due to the favorable growth conditions of AFs-producing species, storage conditions, and agricultural practices [35,36].

The dairy industry in Sri Lanka is supported by several large-scale companies as well as a few small-scale companies [37]. Updating

Table 4
Occurrence of AFM1 in cheese from different countries with their current state of regulation.

Country	Cheese type	Reported values (μg/kg)	Regulator limit set by the country	Reference
Developed countries				
South Korea	Cheese	0.015–0.136	AFM1 for milk 0.5 μg/kg and no legal limits for cheese (Korea Food and Drug Administration (KFDA 2003)	[43]
Italy	Italian cheese	0.250	AFM1 for cheese 0.45 μg/kg (Italian Health Department 2004)*	[44,45]
Developing countries				
Turkey	Industrial Halloumi cheese,	0–0.016	AFM1 for cheese 0.5 μg/kg (Turkish Food Codex, TFC 2008)	[29]
	homemade Halloumi cheese	0–0.005		
Pakistan	White cheese	0.004–0.600	No legal limits for milk or dairy products	[46]
	Cheese cream	0.004–0.500		
India	Cheese	0.005–1.968	AFM1 for milk and dairy products 0.5 μg/kg (Food Safety and Standards Authority of India (FSSAI 2011)	[47]
China	Cheese	0.160–0.320	AFM1 for milk 0.5 μg/kg (China legal limit for milk)	[35]
Egypt	Soft Cheese	0.050–0.097	AFM1 for cheese 0 ng/kg (Egyptian regulation 1990)	[48]
Yemen	Cheese	0.255–5.955	No legal limits for milk or dairy products	[49]
Brazil	Minas Frescal Cheese	0.113 ± 0.092	AFM1 for cheese 2.5 μg/kg (Brazilian MPL cheese, ANVISA 2011)	[36]
Mexico	Oaxaca Cheese	0.020–22.800	Mexico does not have a regulatory limit for the AFM1 concentration in cheese	[50]
Iran	White cheese	0.041–0.374	AFM1 for cheese 0.2 μg/kg (Institute of Standards and Industrial Research of Iran (ISIRI 2002)	[51]
Iran	White cheese	0.093–0.309	AFM1 for cheese 0.2 μg/kg (Institute of Standards and Industrial Research of Iran (ISIRI 2002)	[52]
	Lighvan cheese	0.071–0.203		
Lebanon	Akkawi cheese	0.053 ± 2.81	AFM1 for milk 0.05 μg/kg same as the European Commission maximum limit	[53]
	Halloumi cheese	0.035 ± 2.81		
	Karishe cheese	0.026 ± 2.72		
	Shanklish cheese	0.044 ± 2.81		
Sri Lanka	Different cheese products	0.110–14.430	AFM1 for milk and dairy products 1 μg/kg (Regulations gazetted in 1993 under the Food Act 1980)	Present study

regulations on maximum limits set for AFM1 in dairy products to align with international standards in a timely manner is a crucial aspect of regulatory governance. Further, it is important to establish regulations to control AFs in animal feed which will support the efforts to minimize AFM1 contamination in milk and dairy products. In Sri Lanka, there is no regulatory limit established for AFB1 contamination in compound feed. Good management practices are required to lower AFB1 contamination in feedstuff during the pre-harvest and post-harvest stages of raw materials, as well as during the production process until the feed reaches the animals [38,39].

Consumers in developing nations commonly encounter challenges with food safety and food security [40]. Significant cases of aflatoxin poisoning incidents are reported in developing nations and the documentation of HCC from prolonged exposure to aflatoxins is extensive and well-established [22] and nearly five billion people in developing countries are exposed to uncontrollable aflatoxins [41]. Only a handful number of studies have explored the contamination of cheese with AFM1 in South Asian countries and these countries showed high levels of AFM1 contamination in cheese products compared to data obtained from developed countries (Table 4). Some countries have established regulatory limits for the AFM1 in raw milk, but they do not have corresponding regulations for dairy products. The present study reports the highest occurrence values in cheese in the Asian continent according to the available information.

In some countries, the existing regulatory limits for AFM1 in cheese are outdated, and in other countries, there are no specific regulations governing AFM1 in cheese at all. Lack of proper regulation and oversight of the dairy industry by the regulators and lack of resources seem to be the key factors for AFM1 contamination of dairy products in developing nations [42]. The establishment of maximum limits for AFM1 is indicative of a country's ability to execute comprehensive quality assurance initiatives throughout the entire dairy food production process. These regulatory limits also reflect the nation's economic resources, technological capabilities, scientific expertise, and managerial proficiency which are essential for executing official oversight and control measures. Studies done in developing countries highlight the necessity of proper regulations of AFs in animal feed and milk/dairy products. Unfortunately, many developing countries have yet to establish maximum limits for the presence of aflatoxins (AFs) in food, animal feed, and aflatoxin M1 (AFM1) levels in milk and dairy products.

Even though the study reports an important food safety issue in the developing world under reproducible and reliable laboratory conditions, there were some limitations associated with the study. The number of samples analyzed was limited due to the limited availability of diverse cheese types under different brands in the Sri Lankan market.

4. Conclusions

Most of the analyzed cheese products available in the Sri Lankan market were contaminated with AFM1 and posed health risks to consumers, especially children of wealthy and middle-income families. Therefore, it is important to establish regulations under the Food Act, No. 26 of 1980 on par with international standards and conduct surveillance to monitor the compliance of products in the market. Milk contamination can be minimized directly by monitoring the AFM1 content inmilk or indirectly by reducing AFB1 contamination in animal feed.

Data availability

Data associated with the study has not been deposited into a publicly available repository and will be made available on request.

CRediT authorship contribution statement

Asanka Mudannayake: Writing – original draft, Resources, Project administration, Methodology, Formal analysis, Data curation. **Sachini Karunarathne:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis. **Pasindu W. Jayasooriya:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Diani Nanayakkara:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Ayesh Abesooriya:** Writing – original draft, Resources, Methodology, Formal analysis. **Susil Silva:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Ruchika Fernando:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of AI and AI-assisted technologies in the writing process

The authors declare that they have not used any AI or AI assiated technologies in writing the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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