Article

The Presence of Aflatoxin M$_1$ in Milk and Milk Products in Bangladesh

Abu Hasan Sumon [1], Farjana Islam [1], Nayan Chandra Mohanto [1], Rahanuma Raihanu Kathak [1], Noyan Hossain Molla [1], Sohel Rana [2], Gisela H. Degen [3,†] and Nurshad Ali [1,*]

[1] Department of Biochemistry and Molecular Biology, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh; hasansumon008@gmail.com (A.H.S.); farjana-bmb@sust.edu (F.I.); ncmohanto@gmail.com (N.C.M.); rahanumaraihanu@gmail.com (R.R.K.); nayanmuntasir@gmail.com (N.H.M.)

[2] Department of Veterinary and Animal Science, Rajshahi University, Rajshahi 6205, Bangladesh; srana31373@gmail.com

[3] Leibniz-Research Centre for Working Environment and Human Factors (IfADo) at the TU Dortmund, Ardeystr. 67, D-44139 Dortmund, Germany; degen@ifado.de

* Correspondence: nali-bmb@sust.edu

† shared senior authorship.

Abstract: As milk provides both micro- and macronutrients, it is an important component in the diet. However, the presence of aflatoxin B$_1$ (AFB$_1$) in the feed of dairy cattle results in contamination of milk and dairy products with aflatoxin M$_1$ (AFM$_1$), a toxic metabolite of the carcinogenic mycotoxin. With the aim to determine AFM$_1$ concentrations in milk and milk products consumed in Bangladesh, in total, 145 samples were collected in four divisional regions (Sylhet, Dhaka, Chittagong, and Rajshahi). The samples comprised these categories: raw milk (n = 105), pasteurized milk (n = 15), ultra-high temperature (UHT)-treated milk (n = 15), fermented milk products such as yogurt (n = 5), and milk powder (n = 5). AFM$_1$ levels in these samples were determined through competitive enzyme-linked immunosorbent assay (ELISA). Overall, AFM$_1$ was present in 78.6% of milk and milk products in the range of 5.0 to 198.7 ng/L. AFM$_1$ was detected in 71.4% of raw milk (mean 41.1, range 5.0–198.7 ng/L), and in all pasteurized milk (mean 106, range 17.2–187.7 ng/L) and UHT milk (mean 73, range 12.2–146.9 ng/L) samples. Lower AFM$_1$ levels were found in yogurt (mean 16.9, range 8.3–41.1 ng/L) and milk powder samples (mean 6.6, range 5.9–7.0 ng/L). About one-third of the raw, pasteurized, and UHT milk samples exceeded the EU regulatory limit (50 ng/L) for AFM$_1$ in milk, while AFM$_1$ levels in yogurt and milk powder samples were well below this limit. Regarding regions, lower AFM$_1$ contamination was observed in Chittagong (mean 6.6, max 10.6 ng/L), compared to Sylhet (mean 53.7, max 198.7 ng/L), Dhaka (mean 37.8, max 97.2 ng/L), and Rajshahi (mean 34.8, max 131.4 ng/L). Yet, no significant difference was observed in AFM$_1$ levels between summer and winter season. In conclusion, the observed frequency and levels of aflatoxin contamination raise concern and must encourage further monitoring of AFM$_1$ in milk and milk products in Bangladesh.

Key Contribution: This study found that AFM$_1$ is frequently present in milk and milk products in Bangladesh and about one-third of the raw, pasteurized, and UHT milk samples exceeded the EU regulatory limit.

1. Introduction

Milk is an important food as it provides micro- and macronutrients essential for the growth and maintenance of human health [1]. As milk and milk products are consumed by all age groups, including young children, dairy milk must be free of toxic compounds, including mycotoxins [2]. However, milk and milk-based products can contain aflatoxin M$_1$

(AFM$_1$), a metabolite of the mycotoxin aflatoxin B$_1$ (AFB$_1$), a potent human carcinogen [3]. AFM$_1$ occurs in the milk of dairy cattle ingesting feed contaminated with AFB$_1$, which is partly converted to this hydroxylated metabolite and then excreted in milk. The fraction of AFB$_1$ in feed that is transferred to milk as AFM$_1$ (carry-over) ranges between 0.6% and 3% with up to 6.2% in high yielding cows [4–6]. Due to its strong toxicity in many species, AFB$_1$ is an undesirable substance in animal feed [7], and its levels in feed for dairy cattle are restricted in several countries in order to minimize carry-over and thereby human AFM$_1$ exposure with milk [1,8].

The presence of AFM$_1$ in milk and dairy products represents a worldwide concern for several reasons: (i) although AFM$_1$ is known to exert lower carcinogenic potency than AFB$_1$, their acute toxicities are rather similar [1,9,10]; (ii) AFM$_1$ is heat stable, and normal processing and storage are not effective in reducing its levels in milk and milk products [11,12]; and (iii) small levels of this contaminant may impose health risks for consumers of large quantities of milk products, such as children, a particularly vulnerable subgroup in the population [13,14].

To protect humans against adverse effects from mycotoxin exposure, regulatory standards (limit values) for aflatoxins as contaminants in food and feed have been set in many countries. For instance, the European Commission (EC) established a maximum limit (ML) of 50 ng of AFM$_1$/kg in milk, and a limit for AFM$_1$ of 25 ng/kg in infant formula and follow-up formula [15], while other regulatory authorities and the Codex Alimentarius Commission have set a higher limit of 500 ng/kg in raw milk [16].

Aflatoxin contamination of feed commodities is frequent in some regions of the world, including South and Southeast Asia [17,18]. Bangladesh has a tropical monsoon climate, with clear seasonal variations in rainfall, temperature, and humidity; agriculture is highly dependent on the weather, with crop production mainly in summer and winter [19,20]. As high humidity at harvest and in storage favor the growth of aflatoxin-producing fungi, it is expected that crops in Bangladesh are also affected. Indeed, when aflatoxins were analyzed in cereal samples collected in all Bangladeshi districts and six times during a year, maize had the highest prevalence and level of contamination; contaminant levels were lower, yet still significant, in rice and in wheat, and all samples showed considerable seasonal variability [21]. Another study in Bangladesh reported the presence of AFB$_1$ in pooled samples of commonly consumed food commodities and in poultry feed [22]. However, data on mycotoxins are scarce, in particular in feeds for livestock, a major part of the economy of Bangladesh [23].

For developing countries, where regulation on food and feed contaminants is not in place or not enforced by regular surveillance, monitoring of AFM$_1$ occurrence in milk is recommended, as it is easy to perform and an indicator for dairy feed contamination. Furthermore, as milk and its products are important components of the human diet, the presence of AFM$_1$ in these products may impose health risks for the consumers. This view is supported by a recent, first study on AFM$_1$ contamination of raw milk and processed milk samples, collected at regional small farms and grocery stores in the Dhaka region [24]: AFM$_1$ was detected in more than half (53/100) of the samples, and among these, 75% were above regulatory limits set in Europe for AFM$_1$ in milk, and 43% exceeded the limit set for AFM$_1$ in dairy milk of 500 ng/kg in Bangladesh [25]. The results of this study in the Dhaka region [24] warrant further analysis, including other regions of the country. Furthermore, the frequent presence of AFM$_1$ in the urine of adults and children in Bangladesh [20,26,27] indicates the necessity of monitoring and screening food items that may be possible sources of aflatoxin exposure in the Bangladeshi population. Thus, the objective of our present study was to determine AFM$_1$ concentrations in milk and milk products collected from four major divisional regions in Bangladesh, and to explore some variables which may affect contamination levels in raw milk.

2. Results

2.1. Levels of AFM_1 in Milk and Milk Products

The detection frequencies and contamination levels of AFM_1 in milk (raw, pasteurized, UHT) and in dairy products (milk powder, yogurt) are presented in Table 1 Overall, AFM_1 was detected in 78.6% of all milk and milk products in the range from 5.0 to 198.7 ng/L (mean 51.5 ng/L). AFM_1 was detected in 71.4% of raw milk samples (mean 41.1 ng/L, range 5.0–198.7 ng/L), and in 100% of pasteurized milk (mean 106 ng/L, range 17.2–187.7 ng/L) and UHT milk (mean 73 ng/L, range 12.2–146.9 ng/L). AFM_1 contamination levels were comparatively lower in yogurt (mean 16.9 ng/L, range 8.3–41.1 ng/L) and milk powder samples (mean 6.6 ng/L, range 5.9–7.0 ng/L). In total, 32.4% of milk samples (raw milk 23.8%, pasteurized and UHT milk 73.3%) exceeded the EU regulatory limit (50 ng/kg) for AFM_1 in milk. AFM_1 contamination levels in all yogurt and milk powder samples were well below the EU regulatory limit.

Table 1. Occurrence and levels of AFM_1 in milk and milk products in Bangladesh.

Samples	N	Positive Samples, n (%)	Mean $\pm$ SD (ng/L)	Median (Range) (ng/L)	Above 50 ng/L n (%)
Raw milk	105	75 (71.4)	41.1 $\pm$ 43.4 [a]	23.5 (5.0–198.7)	25 (23.8)
Pasteurized milk	15	15 (100)	106.0 $\pm$ 58.5 [b]	115.7 (17.2–187.7)	11 (73.3)
UHT milk	15	15 (100)	73.0 $\pm$ 42.0 [c]	74.0 (12.2–146.9)	11 (73.3)
Milk powder	5	4 (80)	6.6 $\pm$ 0.5	6.7 (5.9–7.0)	0
Yogurt	5	5 (100)	16.9 $\pm$ 13.6	12.1 (8.3–41.1)	0
Total	145	114 (78.6)	51.5 $\pm$ 50.4	27.9 (5.0–198.7)	47 (32.4)

Only positive samples ($\geq$LOD; 5 ng/L) were considered during mean and median calculation. [a] When raw milk is compared to milk powder ($p < 0.001$), [b] when pasteurized milk is compared to raw milk ($p < 0.01$), yogurt, and milk powder ($p < 0.001$), [c] when UHT milk is compared to yogurt ($p < 0.01$) and milk powder ($p < 0.001$). *p*-values were obtained from one-way ANOVA.

2.2. Regional and Seasonal Variations in AFM_1 Contamination Levels

AFM_1 contamination levels were compared for the raw milk samples collected from the four divisional regions of Bangladesh. The lowest AFM_1 contamination was observed in the Chittagong division (mean 6.6 ng/L, max 10.6 ng/L) compared to Sylhet (mean 53.7 ng/L, max 198.7 ng/L), Dhaka (mean 37.8 ng/L, max 97.2 ng/L), and Rajshahi (mean 34.8, max 131.4 ng/L) division (Table 2). The Sylhet region had the maximum amount of milk samples (32.5%) exceeding the EU regulatory limit, whereas all the samples from the Chittagong division were well below this limit.

Table 2. Levels of AFM_1 in raw milk samples collected from four divisional regions.

Regions	n	Positive Sample n (%)	Mean $\pm$SD (ng/L)	Median (Range) (ng/L)	Above 50 ng/L n (%)
Sylhet	40	34 (85)	53.7 $\pm$ 51.0	34.0 (5.5–198.7)	13 (32.5)
Dhaka	25	15 (60)	37.8 $\pm$ 29.2	33.2 (5.1–97.2)	7 (28.0)
Chittagong	20	8 (40)	6.6 $\pm$ 1.9 *	5.8 (5.0–10.6)	0 (0)
Rajshahi	20	18 (90)	34.8 $\pm$ 39.1	19.2 (6.3–131.4)	5 (25)
Total	105	75 (71.4)	41.0 $\pm$ 43.4	23.5 (5.0–198.7)	25 (23.8)

Only positive samples ($\geq$LOD: 5 ng/L) were considered during mean and median calculation. * When comparing mean AFM_1 concentration to Sylhet ($p < 0.001$), Dhaka ($p < 0.01$) and Rajshahi ($p < 0.05$) regions. *p*-values were obtained from one-way ANOVA.

Regarding seasonal variations, there was no significant difference at the level of AFM_1 contamination between samples collected in the winter (mean 39.9 ng/L, max 115.5 ng/L) or summer (mean 41.6 ng/L, max 198.7 ng/L) period (Table 3). Yet, the percentage of milk samples that exceeded the EU regulatory limit was slightly higher in the summer (26.2%) than in the winter (20.5%) season.

Table 3. Levels of AFM$_1$ in raw milk samples collected in winter and summer seasons.

Season	n	Positive Sample n (%)	Mean ± SD (ng/L)	Median (Range) (ng/L)	Above 50 ng/L n (%)
Winter	44	28 (63.6)	39.9 ± 34.3	27.9 (5.5–115.5)	9 (20.5)
Summer	61	47 (77.1)	41.6 ± 43.4	16.5 (5.0–198.7)	16 (26.2)
Total	105	75 (71.4)	41.0 ± 43.4	23.5 (5.0–198.7)	25 (23.8)

Only positive samples (≥LOD: 5 ng/L) were considered during mean and median calculation.

3. Discussion

As AFM$_1$ may pose a health risk for consumers of milk from dairy cattle fed with AFB$_1$-contaminated feedstuffs, regulatory authorities have established limits for this mycotoxin metabolite (see Introduction). Regular monitoring of AFM$_1$ is needed to control for compliance with food standards and protecting the population against hazardous dietary exposures. The prevalence and levels of AFM$_1$ in milk and dairy products analyzed in various regions of the world (data reviewed by [1,8,28,29]) indicate a wide spectrum of human exposure to AFM$_1$, with considerable differences between climate zones and countries. In the context of present work, there are studies on AFM$_1$ occurrence in milk and milk products from South-Asian countries (listed in Table 4), which include recent ones from Bangladesh.

Table 4. Occurrence and levels of AFM$_1$ in milk and dairy products in South-Asian countries.

Country	Sample Type	Positive Samples n (%)	Mean (ng/L)	Range (ng/L)	Samples ≥ 50 ng/L n (%)	Analytical Method	LOD/LOQ ng/L	References
Bangladesh	Raw Milk	75/105 (71.4)	41.1	5–198.7	25 (23.8)	ELISA (SKM)	5.0/-	Present study
	Pasteurized milk	15/15 (100)	106	17.2–187.7	11 (73.3)			
	UHT milk	15/15 (100)	73	12.2–146.9	11 (73.3)			
	Milk powder	4/5 (80)	6.6	5.9–7.0	0			
	Yogurt	5/5 (100)	16.9	8.3–41.1	0			
Bangladesh	Raw milk	35/50 (70.0)	699.1	22.8–1489.3	34 (97.0)	ELISA (SKM)	18.0/NI	[24]
	Pasteurized milk	13/25 (52.0)	99.8	18.1–672.2	6 (46.0)			
	UHT milk	05/25 (20.0)	35.5	25.1–49.0	0			
India	IMF	17/17 (100.0)	350.0	77.0–844.0	17 (100.0)	ELISA (SKM)	NI	[30]
	Infant formula	17/18 (94.0)	326.0	143.0–770.0	17 (100.0)			
	MBCWF	38/40 (95.0)	267.0	65.0–1012.0	38 (100.0)			
	Liquid milk	4/12 (33.0)	86.0	28.0–164.0	3 (75.0)			
India	Raw milk	110/189 (58.0)	917.0	7.0–13,100.0	96 (50.8)	ELISA (SKM)	5.0/NI	[31]
India	Infant formula	18/18 (100.0)	NI	150.0–713.0	18 (100.0)	ELISA (SKM)	5.0/NI	[32]
	Liquid milk	54/54 (100.0)	NI	172.0–820.0	54 (100.0)			
India	UHT milk	30/45 (66.6)	NI	NI	29 (96.7)	HPLC (IAC)	4.0/NI	[33]
	Raw milk	3/7 (42.9)	NI	NI	NI			
	Pasteurized milk	29/45 (42.9)	NI	NI	3 (10.3)			
India	Buffalo milk	58/150 (38.6)	26.0	NI	9 (16.0)	ELISA (SKM) HPLC (IAC)	5.0/NI (ELISA)	[34]
	Cow milk	68/150 (45.3)	18.0	NI	30 (44.0)			
	Goat milk	50/150 (33.3)	14.0	NI	5 (10.0)			
	Sheep milk	55/150 (36.6)	17.0	NI	7 (12.0)			
	Cheese	2/2 (100.0)	NI	NI	0			
Nepal	Raw milk	8/16 (50.0)	30.0	26.0–138.0	4 (25.0)	TLC	NI	[35]
	Pasteurized milk	6/16 (37.5)	22.0	25.0–127.0	3 (18.8)			
Pakistan	Raw milk	168/168 (100)	199–503	10–700	167 (99.4)	Fluorometric (IAC)	NI	[36]
Pakistan	UHT milk	85 (NI)	254.9	ND-1536.0	80 (94.1)	ELISA (SKM)	4.4/NI	[37]
	Pasteurized milk	78/78 (100.0)	939.5	32.8–4808.0	74 (95.0)			
	Raw milk	55/55 (100.0)	1535.0	1912.0–7460.7	55 (100.0)			
Pakistan	Milk	143/156 (91.7)	346.2	20.0–3090.0	125 (80.1)	ELISA (SKM)	5.0/NI	[38]

<p style="text-align:center">**Table 4.** *Cont.*</p>

Country	Sample Type	Positive Samples n (%)	Mean (ng/L)	Range (ng/L)	Samples ≥ 50 ng/L n (%)	Analytical Method	LOD/LOQ ng/L	References
Pakistan	Raw milk	137/150 (91.3)	198.8	6.0–554.0	108 (72.0)	ELISA (SKM)	4.3/5.0	[39]
	TW	30/30 (100.0)	113.0	13.0–257.0	17 (56.0)			
	UHT milk	30/30 (100.0)	164.0	10.0–345.0	20 (66.0)			
Pakistan	Milk	76/107 (71)	150.1	4.0–845.4	44 (58.0)	HPLC (IAC)	4.0/NI	[40]
	Yogurt	59/96 (61.0)	94.4	4.0–615.8	28 (47.0)			
	Butter	33/74 (45.0)	69.7	4.0–413.4	17 (52.0)			
Pakistan	Raw milk	63/107 (59.0)	55.0	<4.0–980.0	38 (35.5)	HPLC-FLD (IAC)	4.0/12.0	[41]
Pakistan	Milk	76/107 (71)	212.2	4–845.4	44 (58)	HPLC-FLD (IAC)	4/NI	[40]
	Yogurt	59/96 (61)	147.1	4–615.8	28 (47)			
	White Cheese	93/119 (78)	189.1	4–595.4	14 (15)			
	Cheese cream	89/150 (59)	172.9	4–456.3	10 (11)			
	Butter	33/74 (45)	156.3	4–413.4	17 (52)			
Pakistan	Shop milk	137/175 (78.0)	176.0	2.0–1600.0	50 (28.6)	ELISA (SKM)	2.0/NI	[42]
	Household milk	25/40 (62.0)	470.0	3.0–1900.0	18 (45.0)			
	Farm milk	15/17 (88.0)	110.0	2.0–794.0	7(41.0)			
	Sweets	134/138 (97.0)	480.0	10.0–1500.0	108 (78.0)		10.0/NI	
Pakistan	Milk	18/21 (85.7)	NI	11.0–14.0		ELISA (SKM)	NI	[43]
	Yogurt	6/10 (60.0)	NI	10.0–13.0	0			
	Butter	4/10 (40.0)	NI	7.0–7.4				
Sri Lanka	Milk	29/87 (33.3)	40.2	13.1–84.5	8 (9.2)	HPLC (IAC)	10.0/NI	[44]

NI: not indicated, UHT: ultra-high temperature, IMF: infant milk food, MBCWF: milk based cereal weaning food, TW: tea whitener, ELISA: enzyme-linked immunosorbent assay, HPLC-FLD: high pressure liquid chromatography with fluorescence detection, SKM: skim milk, IAC: immunoaffinity columns. Note: for cheese, EU limit is 250 ng/kg.

Regulation on mycotoxin contamination of major food categories, and limits for AFM_1 in milk, were issued in 2017 in Bangladesh [25], yet data from related monitoring are recently emerging. The new data presented here on AFM_1 in milk and milk products show a high prevalence of this contaminant (78.6%) in all samples collected in the four divisional regions in Bangladesh (Table 1). Detection frequency and mean levels of AFM_1 in pasteurized and UHT milk were higher than those in raw milk, yet the concentration ranges observed in these sample types (5–198 ng/L) were similar. This observation is in accordance with findings that heat treatments in pasteurization and sterilization do not cause notable changes in AFM_1 content in such products [1,8]. On the other hand, AFM_1 contamination appeared to be lower in milk powder and yogurt (range 5.9–41.1 ng/L), but the sample numbers analyzed here preclude further conclusions on the effects of processing, although it has been found that AFM_1 levels can decrease in yogurt production [14,45].

The higher number of raw milk samples allowed us to explore possible regional differences in AFM_1 occurrence. The lowest AFM_1 prevalence and levels were observed in milk from the Chittagong division (Table 2) with none of the samples exceeding the EU regulatory limit of 50 ng/kg. In contrast, in the Sylhet, Dhaka, and Rajshahi divisions, 32.5%, 28%, and 25%, respectively, of the collected milk had AFM_1 levels exceeding this standard. Cattle feeding and grazing practices can vary between small farmers and commercial dairy farms. Small farmers are accustomed to open grazing practices of their cattle. In addition, they use stored feed such as dry rice paddy straw, rice husk, and wheat bran. Larger farms usually fully depend on stored feed such as dry rice paddy straw, rice husk, wheat bran, maize, different types of pulses, mustard oil cake, soybean bush, and, to a lesser extent, grass. Due to limited resources for this study, we could not collect background information on farming practices and dairy feeds (green fodder, dry fodder, and concentrate) provided at the different locations. Thus, we presently have no information on differences in AFM_1 contamination. Yet, plans exist to investigate this aspect further, and to raise awareness on critical aspects such as proper storage of dairy feed and other relevant aspects in mycotoxin contamination [1,31].

Regarding seasonal variations, there was no significant difference in the levels of AFM_1 contamination between the winter and summer period (Table 3), although the percentage of raw milk samples that exceeded the EU regulatory limit was slightly higher in the summer (26.2%) than in the winter (20.5%) season. Our findings differ from studies in Pakistan and India, at which the AFM_1 levels in milk were often higher in the rainy/monsoon seasons and lower in the summer [31,36,46,47]. These outcomes are related to various factors, namely environmental conditions (high humidity) conducive for growth of *Aspergillus* in stored feed, and dairy animals getting more compound-feed in winter or more out-pasturing in summer. Yet, in these studies [31,36,46,47], the mean AFM_1 milk levels in both seasons were high compared to those found in our study in Bangladesh.

Recently, Tarannum et al. [24] analyzed samples collected in the Dhaka district of Bangladesh and reported higher ranges and mean AFM_1 concentrations in raw milk, while processed (pasteurized, UHT) milk had similar levels of contamination as observed in the present study (Table 4). Thus, both studies document frequent contamination of dairy milk in Bangladesh. Variations in AFM_1 milk levels have been observed in other countries where multiple studies of this type have been conducted, as in Pakistan and India (Table 4). Such differences observed in the prevalence of AFM_1 levels may depend on several factors, such as environmental conditions, different farming and feeding practices, and the quality and safety control system of the food business operators concordant with the legislations in force [1,31,36].

While the studies listed in Table 4 differ in milk types and dairy products covered, sample sizes, and methods used for AFM_1 analysis, the reported outcomes show the frequent occurrence of the mycotoxin metabolite in food intended for human consumption in South Asia. AFM_1 levels in a high percentage of samples were found to exceed the limit of 50 ng/kg set in the EU, and many samples crossed the 10-fold higher limit permitted in other regions of the world. In particular, the high AFM_1 levels detected in infant formula

in two studies in India [30,32] and in sweets in Pakistan [42] raise concerns. Thus, as these products are consumed by vulnerable parts of the population, they should be included in future sampling plans for AFM_1 analysis in Bangladesh. AFM_1 levels reported in dairy milk are higher than those determined recently in human breast milk (51.6%, mean 4.42 ng/L) in Bangladesh [48]. Thus, one can conclude that human breast milk is better and safer for infants than dairy milk.

In summary, the present study along with study [24] document the frequent presence of AFM_1 in milk and milk products in Bangladesh, and at levels which often exceed regulatory standards for this toxic contaminant. Therefore, and to protect consumers against potential health risks from exposure to AFM_1, more extensive and periodic control of AFM_1 concentration in milk and dairy products is needed. AFM_1 monitoring is relatively easy to perform, in contrast to an analysis of AFB_1 contamination in animal feeds due to the heterogeneous distribution of the mycotoxin in raw materials and problems derived from sampling procedures [6]. As AFB_1 contaminated feed is the source of AFM_1 in milk, improved feed practices and proper storage conditions must be implemented to keep mycotoxin contamination in animal feedstuffs as low as possible.

4. Conclusions

AFM_1 was frequently detected in milk and milk products collected in four regions of Bangladesh, and at levels that raise concern for the health of consumers, in particular for young children. Regular surveillance and monitoring are needed to prevent and control aflatoxin contamination in milk and milk products in Bangladesh. Furthermore, government agencies should train farmers by raising awareness of the toxicity of aflatoxins, of proper storage conditions of cattle feed to prevent mold growth, and encouraging further studies at dairy companies in order to reduce potential health risks and economic losses.

5. Materials and Methods

5.1. Study Areas and Sample Collection

This study was conducted at the Department of Biochemistry and Molecular Biology, Shahjalal University of Science and Technology, Sylhet, Bangladesh. Between December 2018 and November 2019, a total of 145 cow milk and milk products were collected: raw milk samples were collected from four major divisional regions (Sylhet, Dhaka, Chittagong, and Rajshahi) of Bangladesh, whereas milk products were collected from the Sylhet and Dhaka regions. The samples were purchased to cover the following categories: raw milk (n = 105), fresh pasteurized milk (n = 15), ultra-high temperature (UHT)-treated milk (n = 15), fermented milk products such as yogurt (n = 5), and milk powder (n = 5). Raw milk samples were collected directly from farmers' houses and dairy farms. Pasteurized milk, UHT-treated milk, and fermented milk products were bought from local retail shops; individual production and expiration dates, if provided, were recorded for the collected samples. Raw milk samples were collected in two seasonal periods (summer: March–October and winter: November–February) to check for variations in AFM_1 contamination. Each category of milk samples was purchased in units of at least 250 mL. The collected samples were stored at $-20\,^\circ$C and analyzed within 2 months of collection.

5.2. Sample Preparation

Aflatoxin M_1 concentration in milk and milk products was measured using a commercial enzyme-linked immunosorbent assay (ELISA) (Helica Biosystems Inc., Santa Ana, CA, USA, catalogue no. 961AFLM01M-96). The sample preparation was performed following the manufacturer's instructions. Briefly, raw milk samples were placed at a refrigerated temperature overnight to initiate the coagulation of fat molecules. Pasteurized milk, UHT milk, and yogurt samples were refrigerated for 1–2 h. Then, all samples were centrifuged at 4000 rpm for 10 min at room temperature to induce separation of the upper fatty layer. For milk powder, about 10 g of sample was dissolved in 100 mL of distilled water and stirred with a magnetic stirrer for 5 min and centrifuged to separate the fat layer. The upper

fatty layer was removed by aspiration, and the lower plasma layer of the milk was used in the assay.

5.3. Laboratory Analyses

Standard solutions and prepared samples (200 μL) were added to the precoated ELISA plates in duplicates and incubated for 2 h at ambient temperature. At the end of incubation, the contents of the wells were discarded, and the wells were washed three times with the washing buffer provided with the assay kit. After the washing steps, 100 μL of the conjugate was added to the wells and incubated for 15 min at room temperature. After incubation, the wells were washed and 100 μL of enzyme substrate was added to each well and incubated for 15 min. Following that, 100 μL of stop solution was added to each well and gently mixed. The absorbance of each microwell was measured at 450 nm by using an ELISA reader (Apollo 11 LB 913, Berthold, Germany) within 15 min. AFM_1 concentration in each well was calculated using a semi-logarithmic standard curve (prepared using 0, 5, 10, 25, 50, and 100 pg/mL of AFM_1 solutions), and the mean of the duplicates was used as the final result. To test the accuracy of the AFM_1 estimations, recovery studies were performed by spiking skim milk samples with three different concentrations of AFM_1 (5, 10, and 25 pg/mL). The repeatability at these three spike concentrations showed acceptable precisions for AFM_1 measurements (Table 5). The recovery was 102%, 98%, and 94.8% in the spiked concentration, respectively. The detection limit (LOD) of this ELISA method was 5 pg/mL or 5 ng/L. Samples exceeding the signal for the highest AFM_1 standard concentration (100 pg/mL) were further diluted and re-tested.

Table 5. The assay repeatability for AFM_1 in spiked milk samples.

Spike Level (ng/L)	Repeats (n)	Mean ± SD (ng/L)	Recovery (%)	RSD (%)
5	5	5.1 ± 0.3	102.0	5.9
10	5	9.8 ± 0.2	98.0	2.4
25	5	23.7 ± 0.6	94.8	2.5

5.4. Statistical Analysis

All data were analyzed statistically using IBM SPSS Statistics version 23. Descriptive analysis was conducted to determine mean, median, and interquartile ranges of the analyte. The obtained data are presented as mean ± standard deviation, and ranges, frequency, and percentiles for the parameters. Differences in AFM_1 concentrations between seasons and regions were analyzed by an independent sample t-test. One-way ANOVA was used to compare AFM_1 concentrations in the four divisional regions in Bangladesh. A level of *p*-value < 0.05 was considered statistically significant.

References

1. Iqbal, S.Z.; Jinap, S.; Pirouz, A.A.; Faizal, A.A. Aflatoxin M1 in Milk and Dairy Products, Occurrence and Recent Challenges: A Review. *Trends Food Sci. Technol.* **2015**, *46*, 110–119. [CrossRef]
2. Flores-Flores, M.E.; Lizarraga, E.; de Cerain, A.L.; González-Peñas, E. Presence of Mycotoxins in Animal Milk: A Review. *Food Control* **2015**, *53*, 163–176. [CrossRef]
3. IARC International Agency for Research on Cancer. Aflatoxins. In *Chemical Agents and Related Occupations*; IARC Monogr Eval Carcinog Risk Chem Hum Vol 100F; IARC Press: Lyon, France, 2012; pp. 225–248.
4. Britzi, M.; Friedman, S.; Miron, J.; Solomon, R.; Cuneah, O.; Shimshoni, J.; Soback, S.; Ashkenazi, R.; Armer, S.; Shlosberg, A. Carry-Over of Aflatoxin B1 to Aflatoxin M1 in High Yielding Israeli Cows in Mid- and Late-Lactation. *Toxins* **2013**, *5*, 173–183. [CrossRef]
5. Gonçalves, B.L.; Gonçalves, J.L.; Rosim, R.E.; Cappato, L.P.; Cruz, A.G.; Oliveira, C.A.F.; Corassin, C.H. Effects of Different Sources of Saccharomyces Cerevisiae Biomass on Milk Production, Composition, and Aflatoxin M1 Excretion in Milk from Dairy Cows Fed Aflatoxin B1. *J. Dairy Sci.* **2017**, *100*, 5701–5708. [CrossRef]
6. Rodríguez-Blanco, M.; Ramos, A.J.; Prim, M.; Sanchis, V.; Marín, S. Usefulness of the Analytical Control of Aflatoxins in Feedstuffs for Dairy Cows for the Prevention of Aflatoxin M 1 in Milk. *Mycotoxin Res.* **2020**, *36*, 11–22. [CrossRef]
7. EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain [CONTAM] Related to Aflatoxin B1 as Undesirable Substance in Animal Feed. *EFSA J.* **2004**, *39*, 1–27. [CrossRef]
8. Prandini, A.; Tansini, G.; Sigolo, S.; Filippi, L.; Laporta, M.; Piva, G. On the Occurrence of Aflatoxin M1 in Milk and Dairy Products. *Food Chem. Toxicol.* **2009**, *47*, 984–991. [CrossRef]
9. EFSA. CONTAM Panel: Scientific Opinion—Risk Assessment of Aflatoxins in Food. *EFSA J.* **2020**, *18*, 6040. [CrossRef]
10. Joint FAO/WHO Expert Committee on Food Additives World Health Organisation, Food and Agriculture Organisation of the United Nations and International Programme on Chemical Safety. In *Evaluation of Certain Mycotoxins in Food: 56th Report of the Joint FAO/WHO Expert Committee on Food Additives*; WHO Technical Report Series 906; WHO: Geneva, Switzerland, 2002.
11. Joint FAO/WHO Expert Committee on Food Additives Safety Evaluation of Certain Mycotoxins in Food. Available online: Http://Www.Inchem.Org/Documents/Jecfa/Jecmono/V47je01.Htm (accessed on 6 June 2021).
12. Campagnollo, F.B.; Ganev, K.C.; Khaneghah, A.M.; Portela, J.B.; Cruz, A.G.; Granato, D.; Corassin, C.H.; Oliveira, C.A.F.; Sant'Ana, A.S. The Occurrence and Effect of Unit Operations for Dairy Products Processing on the Fate of Aflatoxin M1: A Review. *Food Control* **2016**, *68*, 310–329. [CrossRef]
13. Sherif, S.O.; Salama, E.E.; Abdel-Wahhab, M.A. Mycotoxins and Child Health: The Need for Health Risk Assessment. *Int. J. Hyg. Environ. Health* **2009**, *212*, 347–368. [CrossRef]
14. Giovati, L.; Magliani, W.; Ciociola, T.; Santinoli, C.; Conti, S.; Polonelli, L. AFM1 in Milk: Physical, Biological, and Prophylactic Methods to Mitigate Contamination. *Toxins* **2015**, *7*, 4330–4349. [CrossRef]
15. European Commission Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Off. J. Eur. Union* **2006**, *364*, 5e24.
16. van Egmond, H.P.; Schothorst, R.C.; Jonker, M.A. Regulations Relating to Mycotoxins in Food: Perspectives in a Global and European Context. *Anal. Bioanal. Chem.* **2007**, *389*, 147–157. [CrossRef]
17. Streit, E.; Naehrer, K.; Rodrigues, I.; Schatzmayr, G. Mycotoxin Occurrence in Feed and Feed Raw Materials Worldwide: Long-Term Analysis with Special Focus on Europe and Asia. *J. Sci. Food Agric.* **2013**, *93*, 2892–2899. [CrossRef]
18. Gruber-Dorninger, C.; Jenkins, T.; Schatzmayr, G. Global Mycotoxin Occurrence in Feed: A Ten-Year Survey. *Toxins* **2019**, *11*, 375. [CrossRef] [PubMed]
19. Ali, N.; Blaszkewicz, M.; Alim, A.; Hossain, K.; Degen, G.H. Urinary Biomarkers of Ochratoxin A and Citrinin Exposure in Two Bangladeshi Cohorts: Follow-up Study on Regional and Seasonal Influences. *Arch. Toxicol.* **2016**, *90*, 2683–2697. [CrossRef] [PubMed]
20. Ali, N.; Blaszkewicz, M.; Hossain, K.; Degen, G.H. Determination of Aflatoxin M1 in Urine Samples Indicates Frequent Dietary Exposure to Aflatoxin B1 in the Bangladeshi Population. *Int. J. Hyg. Environ. Health* **2017**, *220*, 271–281. [CrossRef]
21. Bhuiyan, M.N.H.; Hassan, M.T.; Begum, M.; Ahan, M.; Rahim, M. Occurrence and Seasonal Trends of Aflatoxin in Rice, Maize and Wheat in Bangladesh. *Int. J. Sustain. Agric. Technol.* **2013**, *9*, 8–14.
22. Roy, M.; Harris, J.; Afreen, S.; Deak, E.; Gade, L.; Balajee, S.A.; Park, B.; Chiller, T.; Luby, S. Aflatoxin Contamination in Food Commodities in Bangladesh. *Food Addit. Contam. Part B* **2013**, *6*, 17–23. [CrossRef]
23. Huque, K.; Sarker, N. Feeds and Feeding of Livestock in Bangladesh: Performance, Constraints and Options Forward. *Bang. J. Anim. Sci.* **2014**, *43*, 1–10. [CrossRef]
24. Tarannum, N.; Nipa, M.N.; Das, S.; Parveen, S. Aflatoxin M1 Detection by ELISA in Raw and Processed Milk in Bangladesh. *Toxicol. Rep.* **2020**, *7*, 1339–1343. [CrossRef]
25. BFSA Bangladesh Food Safety Authority. Food Safety Regulations. Available online: Http://Www.Bfsa.Gov.Bd/Sites/Default/Files/Files/Bfsa.Portal.Gov.Bd/Law/81690675_9a69_483b_8915_1fe8f6b2ddd0/Food-Safety-(Contaminants,-Toxins-and-Harmful-Residues)-Regulations,-2017.Pdf (accessed on 18 February 2021).
26. Ali, N.; Hossain, K.; Blaszkewicz, M.; Rahman, M.; Mohanto, N.C.; Alim, A.; Degen, G.H. Occurrence of Aflatoxin M1 in Urines from Rural and Urban Adult Cohorts in Bangladesh. *Arch. Toxicol.* **2016**, *90*, 1749–1755. [CrossRef] [PubMed]

27. Ali, N.; Manirujjaman, M.; Rana, S.; Degen, G.H. Determination of Aflatoxin M1 and Deoxynivalenol Biomarkers in Infants and Children Urines from Bangladesh. *Arch. Toxicol.* **2020**, *94*, 3775–3786. [CrossRef] [PubMed]

28. Womack, E.D.; Sparks, D.L.; Brown, A.E. Aflatoxin M1 in Milk and Milk Products: A Short Review. *World Mycotoxin J.* **2016**, *9*, 305–315. [CrossRef]

29. Rahmani, J.; Alipour, S.; Miri, A.; Fakhri, Y.; Riahi, S.-M.; Keramati, H.; Moradi, M.; Amanidaz, N.; Pouya, R.H.; Bahmani, Z.; et al. The Prevalence of Aflatoxin M1 in Milk of Middle East Region: A Systematic Review, Meta-Analysis and Probabilistic Health Risk Assessment. *Food Chem. Toxicol.* **2018**, *118*, 653–666. [CrossRef] [PubMed]

30. Rastogi, S.; Dwivedi, P.D.; Khanna, S.K.; Das, M. Detection of Aflatoxin M1 Contamination in Milk and Infant Milk Products from Indian Markets by ELISA. *Food Control* **2004**, *15*, 287–290. [CrossRef]

31. Patyal, A.; Gill, J.P.S.; Bedi, J.S.; Aulakh, R.S. Potential Risk Factors Associated with the Occurrence of Aflatoxin M1 in Raw Milk Produced under Different Farm Conditions. *J. Environ. Sci. Health Part B* **2020**, *55*, 827–834. [CrossRef]

32. Kanungo, L.; Bhand, S. A Survey of Aflatoxin M1 in Some Commercial Milk Samples and Infant Formula Milk Samples in Goa, India. *Food Agric. Immunol.* **2014**, *25*, 467–476. [CrossRef]

33. Siddappa, V.; Nanjegowda, D.K.; Viswanath, P. Occurrence of Aflatoxin M1 in Some Samples of UHT, Raw & Pasteurized Milk from Indian States of Karnataka and Tamilnadu. *Food Chem. Toxicol.* **2012**, *50*, 4158–4162. [CrossRef]

34. Nile, S.H.; Park, S.W.; Khobragade, C.N. Occurrence and Analysis of Aflatoxin M1 in Milk Produced by Indian Dairy Species. *Food Agric. Immunol.* **2016**, *27*, 358–366. [CrossRef]

35. Kafle, P.; Sedai, D.; Rai, K.P.; Pokharel, B.B. Study on the Level of Aflatoxin M1 Contamination in Raw and Processed Milk Marketed in Kathmandu Valley. *J. Food Sci. Technol. Nepal* **2012**, *7*, 52–56. [CrossRef]

36. Hussain, I.; Anwar, J. A Study on Contamination of Aflatoxin M1 in Raw Milk in the Punjab Province of Pakistan. *Food Control* **2008**, *19*, 393–395. [CrossRef]

37. Yunus, A.; Imtiaz, N.; Khan, H.; Ibrahim, M.; Zafar, Y. Aflatoxin Contamination of Milk Marketed in Pakistan: A Longitudinal Study. *Toxins* **2019**, *11*, 110. [CrossRef] [PubMed]

38. Asghar, M.A.; Ahmed, A.; Asghar, M.A. Aflatoxin M_1 in Fresh Milk Collected from Local Markets of Karachi, Pakistan. *Food Addit. Contam. Part B* **2018**, *11*, 167–174. [CrossRef]

39. Ahmad, M.; Awais, M.; Ali, S.W.; Ali Khan, H.A.; Riaz, M.; Sultan, A.; Shakeel Bashir, M.; Ishtiaq Chaudhry, A. Occurrence of Aflatoxin M1 in Raw and Processed Milk and Assessment of Daily Intake in Lahore, Multan Cities of Pakistan. *Food Addit. Contam. Part B* **2019**, *12*, 18–23. [CrossRef] [PubMed]

40. Iqbal, S.Z.; Asi, M.R. Assessment of Aflatoxin M1 in Milk and Milk Products from Punjab, Pakistan. *Food Control* **2013**, *30*, 235–239. [CrossRef]

41. Iqbal, S.Z.; Asi, M.R.; Selamat, J. Aflatoxin M_1 in Milk from Urban and Rural Farmhouses of Punjab, Pakistan. *Food Addit. Contam. Part B* **2014**, *7*, 17–20. [CrossRef]

42. Sadia, A.; Jabbar, M.A.; Deng, Y.; Hussain, E.A.; Riffat, S.; Naveed, S.; Arif, M. A Survey of Aflatoxin M1 in Milk and Sweets of Punjab, Pakistan. *Food Control* **2012**, *26*, 235–240. [CrossRef]

43. Maqbool, U.; Anwar-Ul-Haq; Ahmad, M. ELISA Determination of Aflatoxin M_1 in Milk and Dairy Products in Pakistan. *Toxicol. Environ. Chem.* **2009**, *91*, 241–249. [CrossRef]

44. Pathirana, U.; Wimalasiri, K.; Silva, K.; Gunarathne, S. Investigation of Farm Gate Cow Milk for Aflatoxin M_1. *Trop. Agric. Res.* **2010**, *21*, 119. [CrossRef]

45. Govaris, A.; Roussi, V.; Koidis, P.A.; Botsoglou, N.A. Distribution and Stability of Aflatoxin M1 during Production and Storage of Yoghurt. *Food Addit. Contam.* **2002**, *19*, 1043–1050. [CrossRef]

46. Aslam, N.; Tipu, M.Y.; Ishaq, M.; Cowling, A.; McGill, D.; Warriach, H.M.; Wynn, P. Higher Levels of Aflatoxin M1 Contamination and Poorer Composition of Milk Supplied by Informal Milk Marketing Chains in Pakistan. *Toxins* **2016**, *8*, 347. [CrossRef] [PubMed]

47. Iqbal, S.Z.; Asi, M.R.; Jinap, S. Variation of Aflatoxin M1 Contamination in Milk and Milk Products Collected during Winter and Summer Seasons. *Food Control* **2013**, *34*, 714–718. [CrossRef]

48. Islam, F.; Trisha, A.D.; Hafsa, J.M.; Degen, G.H.; Ali, N. Occurrence of aflatoxin M_1 in human breast milk in Bangladesh. *Mycotoxin Res.* **2021**. [CrossRef] [PubMed]