

Aflatoxin M₁ in Breast Milk, Cow Milk and Milk Products in Minna, Nigeria and their Predisposing Factors

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Abstract

Herein is a focus on incidence and levels of Aflatoxin M₁ (AFM₁) in milk of breast feeding mothers, cow milk and vended milk products within Minna, Nigeria obtained by analysis of 140 samples of milk and milk products using high performance liquid chromatography (HPLC). The findings reveal; incidence of 77.5% in human milk, and suggests that diet, socioeconomic status and ethnicity influence exposure. Nomadic cow milk samples had the highest incidence of 80%, cheese had 40% incidence, nono had 35% incidence, commercial cow milk had 25% incidence and yoghurt had 10% incidence. In a total of 69 contaminated samples, 51 were at levels above (0.05 µg/L) the EU and Nigerian legislated limit. Exposure to AFM₁ from milk and milk products at concentrations demonstrated in this investigation is of great concern as infants in the country are encouraged to be exclusively breast fed for six months after which alternative milk intake sometimes from animal origin can be sourced. Established carcinogenicity and growth impairing potentials of AFM₁ stands as sources of concern and hence the need for enforcement of the legislated limit by regulatory agencies in Nigeria.

Keywords: Aflatoxin M₁; Diet; Socioeconomic status; Breast milk; Cow milk; Cow milk products

Introduction

The southern and northern parts of Nigeria, are the two major divisions to which the ecological, political and ethnic sub-divisions are referenced. The southern part dominantly has higher rainfall and soil which favours tuber and oil tree plantation while the northern part has a relatively lower rainfall and ecology which favours the growth of cereals [1]. Most southern Nigerians thereby subsist primarily on tubers while most northern Nigerians subsist primarily on cereals, thus establishing unique dietary affiliations in both cases. Socioeconomic status is another factor that determines food consumption pattern among individuals; it influences purchasing power, choice of food and consumption of food varieties. Both the ethnic and socioeconomic factors influence the diet and nutrition of individuals in Nigeria.

Milk and milk products form a major part of human and animal nutrition globally; it is also a major part of infant formula. Human breast milk contains nutritional factors such as fats, protein, carbohydrates, mineral contents and other bioactive factors such as cells, growth factors, prebiotics, anti-infectious and anti-inflammatory agents [2] for which it is recommended that mothers exclusively breast feed their child in the first six months of their lives [3]. Nigeria recorded about 20 million live cows and produced 570, 000 tonnes of fresh cow milk. Most cows are reared in pastoral systems where they have varying feed source depending on seasonality in Nigeria. Cow milk can be consumed fresh or in other processed forms including; “nono” (Hausa) a naturally fermented (24 hr) and locally pasteurized form of cow milk, cheese locally called “wara” a coagulated form of milk protein and yoghurt a fermented form of milk among others. UNICEF statistics have shown infant (under one) mortality rate and low birth weight in Nigeria to be 7.8% and 15.2% respectively, weight at birth is a good indicator of the mother’s health and nutritional status as well as the newborn’s chances for survival, growth, long-term health and psychosocial development.

Both human breast milk and cow milk can be contaminated with AFM₁, this is because they ingest food and feed which are substrates

for fungi and their toxins. Upon ingestion of food/feed containing aflatoxin B₁, the body attempts to detoxify the toxin and in the process aflatoxin B₁ is hydroxylated to a less toxic metabolite referred to as AFM₁ in the liver. The new metabolite is less toxic, however still have toxic potentials and is being classified as a group 2B carcinogen [4] unlike the parent which is a group 1A carcinogen. AFM₁ is then excreted into blood and is present in milk of both human and animals. The metabolites are transferable to sucking babies as well as calves leading to a myriad of toxic effects such as genotoxic carcinogenesis, liver necrosis, haemorrhage, stunted growth, underweight, reduced food/feed efficiency, immune system suppression, reduced response to vaccines and death [5,6].

Studies on natural exposure to AFM₁ in milk and milk product in Nigeria include; 14.2% and 85% breast milk contamination in South West Nigeria respectively [7-9], 100% prevalence of AFM₁ in fresh milk, fermented defatted skimmed milk (nono) and partially skimmed milk (*kindirmo*) from two dairy farm settlements in Bida, Northern Nigeria at mean concentrations of 0.665, 0.924, and 0.575 µg/L respectively [10], 100% vended milk contamination by AFM₁ (mean = 0.07 µg/L) in Cross River state, Southern Nigeria [11], Contamination of 7 brands (19 samples) of (100) powdered milk collected from Lagos, Southern Nigeria (mean= 0.02 - 0.41(µg/kg)) [12], contamination of 25 milk powder samples (10 brands) imported, branded and sold in Nigerian

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market with AFM₁ level ranging between $0.13 \pm 0.01 - 3.75 \pm 0.01$ ppb in all tested samples [13].

In view of the toxic effects of these toxins, this study was embarked on to determine the incidence and levels of the metabolite in breast milk of nursing mothers, fresh cow milk and milk products including yoghurt, cheese, 'nono' within Minna, Niger state. This has not been done in earlier studies. The study will also identify possible risk factors, and identify relationship between ethnicity, socioeconomic status and diet of the mothers, to AFM₁ levels.

Materials and Methods

Ethical clearance

Clearance was obtained from the Federal University of Technology Minna and Niger State Veterinary Office Minna. All human subjects were made aware of the context and purpose of the study, and if they agreed to participate, a written informed consent was obtained.

Sampling

After administering consent letters and questionnaires to breast feeding mothers, about 30 ml of breast milk was collected into a sterile plastic container and packaged in an ice pack to the laboratory where they were analyzed. Fresh cow milk was obtained from the milk of lactating nomadic cows and cows reared in a closed system for commercial purpose. The cattle were milked from the first drop of the foremilk to the last drop of the stripping. Yoghurt, cheese and 'nono' samples were bought from various purchase points. A total of 140 samples were analyzed including; 40 breast milk, 20 fresh cow milk, 20 commercial cow milk, 20 yoghurt, 20 cheese and 20 nono samples.

Chemicals

Aflatoxin M₁ reference standard was purchased from Sigma-Aldrich, USA. HPLC grade solvents; acetonitrile, chloroform were ordered from Merck, Germany.

Extraction of Aflatoxin M₁

The Association of Analytical Chemists (AOAC) official method 980.21 was used for the extraction of AFM₁ as described by Elzupir et al. Fifteen (15) ml of milk was measured and dispensed into a clean separating funnel to which 40 ml of chloroform and 3 ml of the sodium chloride solution was added. The separating funnel containing the mixture was securely stoppered and shaken gently to enable clear mixture and adequate separation. The clear lower part of the mixture containing the chloroform was eluted into a 250 ml beaker and evaporated to dryness by placing the beaker containing the chloroform into a water bath heated to 50°C. The dry extract was then dissolved in 10 ml of acetonitrile and defatted twice with 15 ml of petroleum ether two times. The petroleum ether layer was discarded, the extract re-dissolved in acetonitrile and transferred into an amber vial. The extract was evaporated to dryness and stored at -20°C in a freezer until further analysis. Upon analysis, the dry film was re-dissolved in 200 µL of the mobile phase (acetonitrile and water 25:75).

HPLC analysis of Aflatoxin M₁

Agilent technologies 1200 High Performance Liquid Chromatography (HPLC) equipped with fluorescent detector (FLD DEABO01608) at excitation and emission wavelength of 365nm and 435nm respectively was used for the AFM₁ determination. The ODS Hypersil 79926OD-564 column, 4 x 125mm with particle size 5 µL in diameter was used at an ambient temperature of 25°C. Acetonitrile:

water in the ratio 25:75 was used at the mobile phase at the flow rate of 0.8 ml min⁻¹. The injection volume was 20 µL. The analysis was carried out with AFM₁ standards (Trilogy analytical laboratory, Washington MO 63090, United States) of known concentrations with AFM₁ eluting a retention time of 4.5 min. Standard solutions of AFM₁ with concentrations of 0.0016, 0.008, 0.04, 0.2 and 1.0 µg L⁻¹ in mobile phase were used to obtain the standard calibration curve. A standard calibration curve with correlation factor of 0.99988 was obtained. The limit of detection for AFM₁ was 0.001 µg L⁻¹. After the HPLC analysis of spiked and unspiked samples, the percentage recovery was calculated was 86.36%

Results

Aflatoxin M₁ content of human breast milk

The Table 1 indicates the level of AFM₁ detected in the breast milk of 40 breastfeeding mothers. The results show a high incidence of AFM₁ contamination in human breast milk (77.5% (31/40)). In addition, 15 out of the 40 samples (37.5%) had levels above the Nigerian and European Union regulated limit of 0.05 µg/L.

Influence of ethnicity, diet and socio-economic status on AFM₁ exposure

Figures 1-3 show Influence of Ethnicity, Diet and Socio-economic status on AFM₁ Exposure.

Our findings (Figure 1) show that mothers of Northern Nigeria origin have significantly higher ($p < 0.005$) incidence 91.7% (22/24) and concentrations (0.1045 ± 0.0270) of AFM₁ in their breast milk than their Southern counterparts 56.3% (9/16) (0.0088 ± 0.0029).

The findings in this work (Figure 2) show that people who subsist more on cereal have significantly higher ($p < 0.05$) levels of AFM₁ (0.1298 ± 0.0309) in comparison to individuals who subsists on other food sources (0.0117 ± 0.0042) and tuber (0.0058 ± 0.0026).

The findings (Figure 3) show that low income earners have a significantly higher ($p < 0.05$) level of AFM₁ (0.1620 ± 0.05513) when compared to the average income earners (0.0408 ± 0.01460) and high income earners (0.0190 ± 0.0076).

Fresh milk from nomadic cows (Table 2) had the highest incidence of contamination (16/20; 80%) within the range of 0.0109-1.3543 µg/L with 15 being above the EU limit. Fresh cow milk from commercial farms contained AFM₁ within the range 0.0464-0.0992 µg/L, 25% (5/20) contamination incidence and 4 samples at unsafe levels.

Nono samples (Table 2) had 7/20 (35 %) contamination incidence, all samples were at unsafe levels appearing within the range 0.2342-1.2516 µg/L and having a mean score of 0.5929 ± 0.0867 . AFM₁ in cheese (Table 2) (8/10) appeared at unsafe levels ($0.1045-1.5302$ µg/L) having a mean score of 0.588 ± 0.1296 . Yoghurt samples had the least contamination incidence 2/20 (10%), the range was (0.5835-0.6470) while the mean concentration was 0.6152 ± 0.0101 .

Discussion

Aflatoxin M₁ in human breast milk

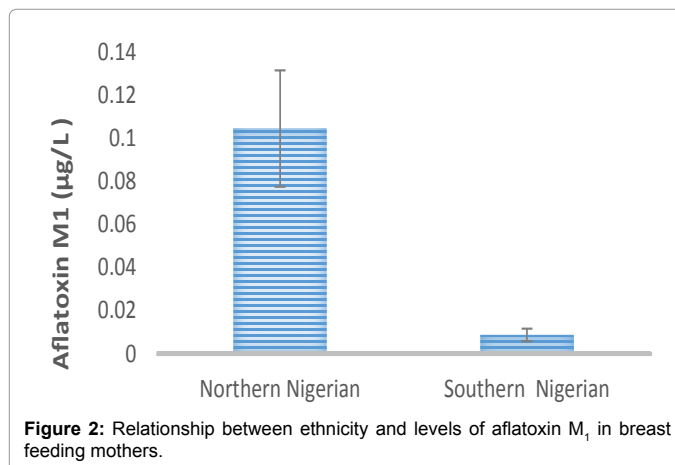
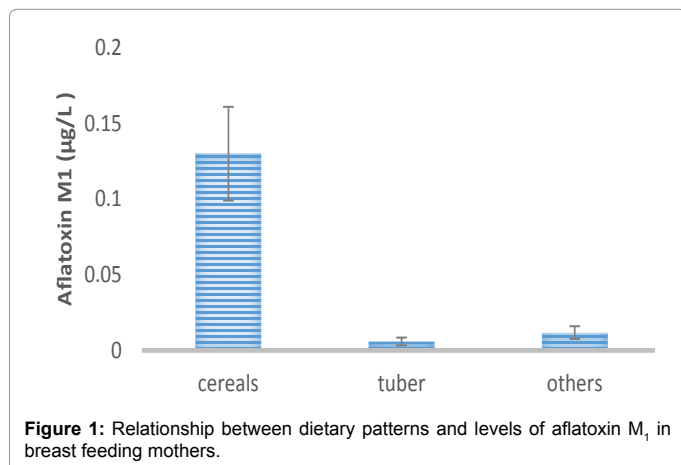
The findings reported in this work for the present of AFM₁ in 31/40 breastfeeding mothers in Minna Nigeria, is similar to an earlier report in another part of the country (Ogun state) which showed 82% contamination of breast milk samples with AFM₁ (3.49 – 35 ng/L) with about 16% exceeding the EU limit [9]. The current report is also

S/N	Diet	Ethnicity	Socio-economic Status	Aflatoxin M1 concentration (µg/L)	Safety Status (0.05µg/L)
1	Cereal	Northern Nigeria	Average	0.0610	Unsafe
2	Cereal	Northern Nigeria	Average	0.0625	Unsafe
3	Cereal	Northern Nigeria	Average	0.0766	Unsafe
4	Cereal	Northern Nigeria	Low	0.6012	Unsafe
5	Cereal	Northern Nigeria	Average	0.0873	Unsafe
6	Cereal	Northern Nigeria	High	0.0638	Unsafe
7	Cereal	Northern Nigeria	High	0.0456	Unsafe
8	Cereal	Northern Nigeria	Low	0.1978	Unsafe
9	Cereal	Northern Nigeria	Average	0.1427	Unsafe
10	Cereal	Northern Nigeria	Low	0.2653	Unsafe
11	Cereal	Northern Nigeria	Low	0.1239	Unsafe
12	Cereal	Northern Nigeria	Low	0.1380	Unsafe
13	Cereal	Northern Nigeria	Low	0.1551	Unsafe
14	Cereal	Northern Nigeria	Average	0.2819	Unsafe
15	Cereal	Northern Nigeria	Low	0.0681	Unsafe
16	Cereal	Northern Nigeria	Average	0.0286	Safe
17	Cereal	Northern Nigeria	Average	0.0065	Safe
18	Cereal	Southern Nigeria	Average	0.0200	Safe
19	Cereal	Southern Nigeria	Low	0.0402	Safe
20	Tuber	Southern Nigeria	Average	0.0010	Safe
21	Tuber	Southern Nigeria	High	0.0000	Safe
22	Tuber	Southern Nigeria	High	0.0179	Safe
23	Tuber	Southern Nigeria	Average	0.0000	Safe
24	Tuber	Southern Nigeria	Average	0.0149	Safe
25	Tuber	Southern Nigeria	High	0.0000	Safe
26	Tuber	Southern Nigeria	Low	0.0121	Safe
27	Tuber	Southern Nigeria	Average	0.0000	Safe
28	Tuber	Southern Nigeria	Average	0.0000	Safe
29	Tuber	Southern Nigeria	Low	0.0187	Safe
30	Tuber	Southern Nigeria	Average	0.0000	Safe
31	Tuber	Northern Nigeria	Average	0.0000	Safe
32	Other	Northern Nigeria	High	0.0270	Safe
33	Other	Northern Nigeria	Average	0.0362	Safe
34	Other	Northern Nigeria	High	0.0012	Safe
35	Other	Northern Nigeria	Average	0.0000	Safe
36	Other	Northern Nigeria	Average	0.0205	Safe
37	Other	Northern Nigeria	Average	0.0168	Safe
38	Other	Southern Nigeria	High	<LOD	Safe
39	Other	Southern Nigeria	High	0.0153	Safe
40	Other	Southern Nigeria	Average	0.0000	Safe

N.B Samples ≥ 0.05 are considered unsafe based on EU legislation adopted in Nigeria.

'Other' refers to individuals whose primary diet is neither cereal nor tuber based, but more of processed foods.

Table 1: Diet, ethnicity, socio-economic status and aflatoxin M1 levels in breast milk of nursing mothers.



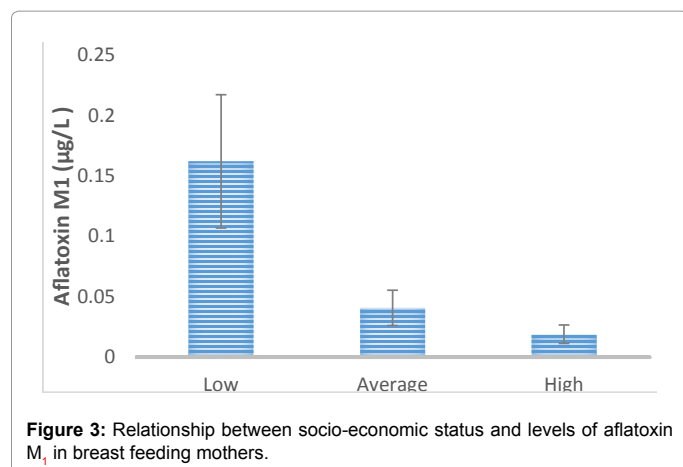


Figure 3: Relationship between socio-economic status and levels of aflatoxin M₁ in breast feeding mothers.

similar to 98% (157/160) breast milk sample contamination with AFM₁ reported in Tehran, Iran [14]. Lower incidence of AFM₁ in breast milk samples have been reported in other parts of the world such as Sari, Iran where only 1 of 136 breast milk sample was contaminated [15] and Brazil where only 2 of 100 samples were contaminated [16]. In most developed countries, mycotoxin legislations are enforced as opposed to most developing countries, other factors that influence variation in survey results from several countries is that of climatic conditions and application of good agricultural practices. In Minna Nigeria most people dominantly consume their traditional foods; sorghum, maize, rice, yam, and millet, these traditional foods are always available all through the year, due to high produce yield and availability of storage methods. It is important to state here that storage factors can potentially add up to the risk factors for AFB₁ contamination of food and feed.

Effects of ethnicity on Aflatoxin M₁: The question being answered here is: what does ethnicity have to do with AFM₁ levels in breast feeding mothers? As clearly stated in the introduction, ethnicity influence diet, mothers of Northern Nigeria origin subsists primarily on cereals while those from Southern Nigeria even though living in the North subsists primarily on tubers; yam, yam flour, yam peel paste (amala), cassava, cassava flour, garri. Our findings show that mothers of Northern Nigeria origin have significantly higher ($p < 0.005$) levels of AFM₁ in their breast milk than their Southern counterparts. A report on the influence of geography on AFM₁ incidence and level was conducted by El-Nezami et al. [17], they determined the prevalence of the toxin in women from low-exposure areas (Victoria, Australia) and those living in assumed high-exposure areas (Thailand), AFM₁ was detected in 11 (28-1031 pg/ml) samples from Victoria and 5 (39-1736 pg/ml) samples from Thailand respectively.

Effects of diet on Aflatoxin M₁: In this work, mothers who subsist more on cereal have significantly higher ($p < 0.05$) levels of AFM₁ in comparison to individuals who subsists on other food sources and tuber. Cereals have proven to be more susceptible to aflatoxins than tubers; cassava and yam. The major cereals in the study area are maize, sorghum, millet and rice. These have been shown from previous reports to harbor aflatoxin B₁; the parent compound of AFM₁ [10,12,18,19]. Prandini et al. [20] established that the presence of Aflatoxin B₁ in corn grain is a risk factor for the appearance of the AFM₁ metabolite in milk. As temperature and humidity changes, expression of different fungi metabolites are either inhibited or favoured in both field and storage conditions. Minna has a relative humidity which ranged between 76% to 88%, and average temperature of 28.3°C which approximate the optimal conditions for aflatoxin synthesis.

Effect of socioeconomic status on Aflatoxin M₁: The respondents were rated based on their average daily purchasing power (> 5 USD, $\geq 2 \leq 5$ USD, $\geq 0 < 2$ USD) as high, average and low income earners respectively. It was observed that the high income earners have higher purchasing power which enables them enjoy (additional) food varieties as opposed to the former. The findings show that low income earners have a significantly higher ($p < 0.05$) level of AFM₁ when compared to the average income earners and high income earners. Adejumo et al. [9] had established that socioeconomic status of mothers significantly influence their dietary exposure and exposure risk of sucklings to AFM₁. This finding thus recommends diversification of food which will provide a balance between susceptible and unsusceptible crops as against monolithic dietary culture on susceptible crops.

Safety concerns of human breast milk: With regards to safety, 15/40 (37.5%) breast milk samples where above the EU MPL of 0.05 µg/L in milk and is considered a potential risk factor for the suckling babies. The degree of exposure of suckling babies to AFM₁ from contaminated mother (s) milk, will be between 8.28 - 15.48 µg of AFM₁ in 6 months (180 days) if world health organization's recommended exclusive breast feeding is adhered to (0.046 - 0.086 µg/day). Assumptions being that a child consumes between 0.7-1.3L of breast milk daily and every 1 L contain an average of 0.0662 µg AFM₁ as reported in this study (Table 2). Bhat and Vasanthi [6], emphasized that children exposed to aflatoxins may become stunted, are underweight, and more susceptible to infectious diseases in childhood and later life. A study carried out in Benin and Togo shows a direct relationship between AFs and underweight status in children [5], while another South African study show the relationship between AF and protein-energy malnutrition condition of kwashiorkor children [21,22].

AFM₁ in milk products

Fresh nomadic cow milk from cows that graze uncontrollably and unrestricted had the highest incidence of contamination (80%) and all the contaminated samples except one i.e 15/20 were at unsafe levels. This is indicative that nomadic cows are exposed to high levels of aflatoxin B₁ in their feeds; which include grasses (majorly *Afzelia africana*, *bamboo*, *Khaya senegalensis*, *Adenolichos paniculatus* and *Mucuna spp*, crop residues (sorghum, millet, rice, soybean) and herbaceous plants [23]. These category of cows are the major sources of beef and local milk mostly consumed in Nigeria. Free grazing cows in Abeokuta Southern Nigeria were reported to have 75% contamination (9.0 to 456.0 ng/L) [8]. In the northern center of Morocco raw milk samples had 13 of 48 (27%) samples contaminated with AFM₁ (10 - 100 ng/L) of which 8% were above the European legislative limit [24], the study also suggested a link between feeding practices, such as the use of silage and AFM₁ contamination.

Fresh cow milk from commercial farms had 25% (5/20) contamination incidence, 4 of the samples were at unsafe levels. The commercial cows are usually kept within a confined grazing area and sometimes fed commercial feed such as wheat ofal, cassava peel, maize chaff and yam peel. A similar study by Bilandžić et al. [25] reported AFM₁ levels in cow milk within the ranges 3.65-162.3 ng/L from eastern Croatia and 2.69 - 44.9 ng/L from other regions of Croatia, in eastern Croatia, 6.7% of cow milk contained AFM₁ levels exceeding the EU limit, they attributed contamination to the use of contaminated supplementary feedstuff in some farms during the study period. Asia et al. [26] found higher AFM₁ levels in milk of animals fed mainly on concentrate mixtures (buffalo and cow) than in other species grazing on fresh greens, they reported 56% and 33% contamination of winter and summer milk samples respectively exceeding EU maximum limit.

Sample	Incidence	Number of samples Above EU action level (0.05 µg/L)	Range (µg/L)	Mean±SEM (µg/L)
Breast Milk	31/40 (77.5%)	15	0.0010 - 0.6012	0.0662 ± 0.0178
Nomadic cows	16/20 (80%)	15	0.0109 - 1.3543	0.5308 ± 0.0938
Commercial cows	5/20 (25%)	4	0.0464 - 0.0992	0.0584 ± 0.0052
Nono`	7/20 (35%)	7	0.2342 - 1.2516	0.5929 ± 0.0867
Yoghurt	2/20 (10%)	2	0.5835 - 0.6470	0.6152 ± 0.0101
Cheese	8/20 (40%)	8	0.1045 - 1.5302	0.588 ± 0.1296

Table 2: Occurrence of aflatoxin M1 in breast milk, cow milk and milk products in Minna, Nigeria.

Nono samples had 35 % (7/20) contamination at unsafe levels. Okeke et al. [10] had reported 100% (10/10) contamination of nono sample in Bida a town bordering Minna, this indicates that the populace that patronize the Fulani nono sellers are at risk of consuming AFM₁ and that fermentation process leading to the production of nono from fresh cow milk do not completely prevent contamination by AFM₁. Juodeikiene et al. [27] have reviewed and shown positive results for the decontamination of various mycotoxins during fermentation processes by bacteria, yeasts, fungi and enzymes. In another study with UHPLC–HESI-MS/MS, Škrbića et al. [28] established that 76% (38/50) milk sample in Serbian market exceeded the EU limit with domestically produced milk having the highest level of AFM₁.

All cheese samples contaminated with AFM₁ (8/10) were unsafe with reference to the EU action level. The cheese processing method may be responsible for further contamination of the samples, this assumption is valid because processing is done in an unstandardized manner. Liquid chromatography coupled with tandem mass spectrometry method detected no AFM₁ contamination in 86 samples of cheese from 15 different countries [29].

In this study, nomadic cow milk and its products; nono and cheese appears to have higher incidence of AFM₁ than commercial cow milk and yoghurt (P<0.05), this is however not in conformity with well-established thoughts as it is expected that grazing animals exposed to grasses are less susceptible to aflatoxin contamination than animals in commercial farms exposed to commercial feeds. This could be due to exposure of the grazing animals to aflatoxin in crop residues and a fairly good feed storage practice by the commercial farms.

The mandate of assuring quality of processed and packaged food in Nigeria belongs to the National Agency for Food, Drug Administration and Control (NAFDAC). This agency occasionally tests packaged food items for compliance to standards, in the case of yoghurt, AFM₁ is also screened for. There has been a general non-compliance among food processors because most of them do not include such screening as part of their quality control process probably for cost related reasons. The yoghurt samples in this work were purchased from commercial vendors, analysis show that only 10% (2/20) of samples had detectable levels of AFM₁ although at unsafe levels. The yoghurt samples are product of a commercial farm in Minna metropolis. This study to a large extent validates the importance of the source of milk used in yoghurt manufacturing and animal feed source.

Conclusion

The finding in this article suggests that, ethnicity, dietary practice and socioeconomic status influences exposure to mycotoxin. Mothers who subsists more on cereals were more at risk of aflatoxin exposure, also the low income earners whose purchasing power is less than \$2 USD daily were more exposed to AFM₁. All sample types including human and cow milk analyzed showed presence of AFM₁. Sixty nine out of a hundred and forty samples analyzed (49.3%) were contaminated, fifty one (36.4%) were unsafe for consumption based on the EU regulation.

It is important to establish proper management technologies targeted at reducing both human and animal exposure to mycotoxins. It is also important for legislative and regulatory bodies charged with food safety responsibilities to actively participate in alleviating the current challenge. Global and regional programs targeted at improving livelihood of poor and disadvantaged populace such as the world feeding programme will do well to provide food diversification, to reduce the exposure to mycotoxin due to monothelic source of feeding. Any attempt to reduce the levels of AFM₁ found in breast or animal milk should emphasize on the reduction of levels of AFB₁ contaminating food/feed, this is practicable because a linear relationship have been established between the concentration of AFM₁ in milk and AFB₁ consumed in feed.

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