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REGULAR ARTICLE

OCCURRENCE OF AFLATOXIN M_1 IN RAW ANIMAL MILK AND DAIRY PRODUCTS IN NORTHERN NIGERIA

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ABSTRACT

A total of 144 samples of milk and milk products comprising 23 raw camel milk, 77 cow milk, 24 goat milk and 20 *kindirmo* (fermented milk) were randomly purchased across four states in northern Nigeria during July 2020 and screened for aflatoxin M1 (AFM1) using an Enzyme-linked immunosorbent assay method. The incidence (and mean values) of AFM_1 in the camel milk, cow milk, goat milk and *kindirmo* samples were 74 % (38 ng/L), 99 % (92 ng/L), 100 % (112 ng/L) and 100 % (145 ng/L), respectively. The mean AFM_1 levels in 22 %, 42 %, 83 % and 50 % of the camel milk, cow milk, goat milk and *kindirmo* samples, respectively, exceeded the European Union threshold of 50 ng/L. Results from this study suggest that consumption of raw animal milk and its products could be a contributing factor to aflatoxin exposure among households in northern Nigeria.

Keywords: Camel, Cow, ELISA, Goat, *Kindirmo*, Yoghurt

INTRODUCTION

Raw milk is a nutrient rich, food-grade liquid regarded as an important source of dietary energy, fats and essential proteins **(Guetouache *et al*., 2014)**. Similar to other countries across the globe, raw animal milk and its traditionally fermented products such as yoghurt [e.g. *Kindirmo* (Figure 1)] and cheese (e.g. wara) serve as important food sources in Nigerian households **(Akinyemi *et al*., 2021)**.

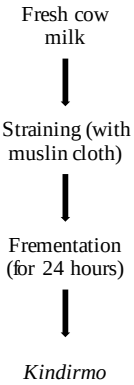

Figure 1: Flow chart for producing *Kindirmo*, a traditionally fermented yoghurt in Nigeria.

According to available reports, about 537 million tons of cow milk was produced in Nigeria in 2018 **(FAOSTAT, 2020)**, but data on the production of other animal milk types consumed within the country is scarce. Nevertheless, in Nigeria, milk from camel, cow, goat, sheep and milk products are consumed by all age groups including infants and children **(Ebringer *et al*., 2008; Oluwafemi and Lawal, 2015; Sudi *et al*., 2011)**. These foods are very affordable and readily available in convenience stores and road side shops, where they are vended **(Fakayode *et al*., 2012)**. Despite its widespread consumption, there is lack of regulation on raw animal milk and its products available for local consumption. As such, there are concerns about the safety of these foods. Of particular importance is the potential contamination of these raw animal milk by aflatoxins, a toxic metabolite of toxigenic strains within *Aspergillus* section *Flavi* **(Frisvad *et al*., 2019)**.
Aflatoxins (AFs) are known carcinogens and can exhibit other toxicological properties such as hepatotoxicity, immunosuppression, mutagenicity and teratogenicity **(International Agency for Research on Cancer, 2015)**. There are approximately 20 types of aflatoxins, however, the most relevant to public health are AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2 **(Chu, 1991; Kumar *et al*., 2017)**. With respect to milk and its product, AFM_1 is the most toxicologically relevant, as such, has received much research attention **(International Agency for Research on Cancer, 2015)**. Aflatoxin M_1, a class 2B human carcinogen, is formed by hydroxylation of AFB_1 (class I human carcinogen) in the liver of ruminants fed with AFB_1-contaminated feed and subsequently excreted into animal products such as milk, meat and urine **(Flores-Flores *et al*., 2015; International Agency for Research on Cancer, 2015)**. Due to its harmful effects, several countries and organizations have set allowable maximum residue levels of AFM_1 in milk, ranging from 0 (in Egypt), through 20 ng/L (Saudi Arabia), 50 ng/L (Brazil, Iran, European Union and South Africa) to 500 ng/L (China, Pakistan and USA) in order to minimize the risk of AFM_1 contamination **(Van Egmond and Jonker, 2004)**.
In the Nigerian market, pasteurized, extended shelf-life and ultra-heat treated milk products are widely available. However, these products are often not affordable to low-income households and are largely restricted to urban areas. Hence, low-income households, mostly in rural areas, depend on raw animal milk for consumption and production of traditional dairy products vended at dairy markets **(NDDP, 2018)**. Despite available data on AFM_1 contamination of animal milk and its products globally **(Aslam and Wynn, 2015; Demissie, 2018; Ketney *et al*., 2017)** including Nigeria **(Makun *et al*., 2010; Okeke *et al*., 2012; Oluwafemi and Lawal, 2015)**, there is paucity of data on AFM_1 in goat milk and camel milk. Additionally, recent data on AFM_1 contamination of cow milk and kindirmo is lacking.
A joint survey by the Nigerian National Bureau of Statistics and Federal Ministry of Agriculture revealed that the northern region is the hub for animal milk production in Nigeria **(NASS 2011)**. In addition, there are indications of widespread exposure to aflatoxins in this region, owing to quantification of copious number of aflatoxins in foods **(Ezekiel *et al*., 2019; Ogara *et al*., 2017; Onyedum *et al*., 2020)** and urine **(Ezekiel *et al*., 2014)**. Thus, investigating aflatoxin contents of animal milk and its product from northern Nigeria is a worthwhile venture. This study was therefore carried out to screen animal milk and its products in northern Nigeria for the presence of AFM_1 using a rapid and cost-effective Enzyme-linked Immunosorbent Assay (ELISA) method.

MATERIALS AND METHODS

Study area

This study was carried out in four states representing the North-east (Bauchi), North-west (Kaduna, Katsina) and North-central region (Plateau) of Nigeria. These states were purposively selected for this study based on the high number of bovine and camel breeders in addition to their status as major dairy producing states in Nigeria.

Sample collection

A total of 144 raw milk and milk product samples were randomly purchased from dairy markets in the four locations in July 2020. Specifically, camel milk (n = 23), cow milk (n = 77), goat milk (n = 24) and *Kindirmo* (n = 20) samples were purchased. The distribution of samples by state include: camel milk, goat milk and *Kindirmo* from Katsina state, and cow milk from Kaduna (n= 24), Katsina (n = 23), Jos (n = 23) and Bauchi (n = 7) states. At the time of sampling, only cow milk was available in all four states.

The milk samples were purchased immediately the animals were milked within the dairy markets situated in the states. Approximately, 20 mL of each sample of milk and milk products were collected in sterile 25 mL universal sample collection bottles and transported to the laboratory in refrigerated (4 °C) boxes for aflatoxin analysis.

Analysis of milk AFM_1 by ELISA

AFM_1 in animal milk and its products was assayed by a quantitative Aflatoxin M_1 ELISA method using the HELICA AFLM01C-ULTRA kits (Helica Biosystems, Inc., USA). All reagents and dairy samples were allowed to thaw to ambient temperature. PBS-Tween provided with the kit was reconstituted in one litre of distilled water. Milk samples were defatted by centrifugation at 2000×g for 5 mins. After centrifugation, the upper fatty layer was removed by aspiration and 450 µL of the lower plasma was applied in the assay. Skimmed milk (1 mL) was added to *kindirmo* samples (1 g) and vortexed for 30 secs and used in the assay according to the manufacturer's instruction.

Using a multichannel micropipette, 200 µL of each sample and AFM_1 standards (0, 10, 30, 80, 200 and 500 pg/mL) were added to appropriate antibody-coated wells and incubated at ambient temperature (25 °C) for 20 mins. During the incubation of the antibody-coated wells, a different set-up was prepared in mixing wells. Briefly, 150 µL of each sample and AFM_1 standards were dispensed into the appropriate mixing wells, 150 µL of conjugate (horseradish peroxidase in buffer) was added into each mixing well and the contents primed by a pipettor five times. After 20 mins of incubating the antibody-coated wells, the contents were discarded and residual standards and samples in the wells were removed by tapping gently on absorbent papers. Thereafter, 100 µL of the contents prepared in the mixing wells were transferred to the corresponding antibody-coated wells and incubated in the dark at ambient temperature for 10 mins. After incubation, the content of each microwell was discarded and the microwells were washed five times with PBS Tween wash buffer. Residual wash buffer was removed by tapping the microwells gently on absorbent papers. Aliquots (100 µL) of the enzyme substrate (tetramethylbenzidine) was then added to each well and the mixture was incubated in the dark at ambient temperature for 10 mins. Finally, a stop solution (100 µL) was added to the wells and the optical densities (OD) of the reaction solution in the microwells were measured on a microplate reader (LABTRON LMPR-A30, United Kingdom) at 450 nm.

In order to estimate the corresponding AFM_1 concentration in each well, a dose-response curve was constructed using the OD values expressed as a percentage of the OD of the zero standards against the AFM_1 content of the standard (as given in the equation below). Unknown samples were measured by interpolation from the standard curve. The recovery (%) of the assay was tested by spiking blank samples at five concentration levels (0, 10, 30, 80, 200, 500 ppb). Recovery ranged between 79 – 113 %.

$$\%Absorbance = \frac{Absorbance\ standard\ (or\ sample)}{Absorbance\ of\ zero\ standard} \times 100$$

Statistical analysis

The IBM Statistical Product and Service Solutions (SPSS) version 21 software was used for data analysis. All results are expressed as the mean ± standard deviation (SD), median, minimum and maximum concentrations of AFM_1. One-way analysis of variance (ANOVA) was used to separate significantly different means where the level of statistical significance was set at $p < 0.05$.

RESULTS

Occurrence of AFM_1 in animal milk and *kindirmo*

Globally, AFM_1 is the only regulated aflatoxin in milk and Nigeria adopts the EU maximum acceptable limit (MAL) of 50 ng/L **(European Union Commission, 2019)**. Thus, this value will be referenced in further discussions in this study.

Overall, the percentage incidence (and mean levels) of AFM_1 detected in camel, cow, goat milk and *kindirmo* samples in this study were 74 % (38 ng/L), 99 % (68 ng/L), 100 % (112 ng/L) and 100 % (145 ng/L), respectively (Table 1). The observed incidence of AFM_1 contamination in cow milk in the present study is similar to the 100 % incidence previously reported in Nigeria **(Oluwafemi and Lawal, 2015)**, Croatia **(Bilandžić *et al.*, 2014)**, Iran **(Khosravi *et al.*, 2013)** and South Africa **(Mulunda, 2016)** albeit higher than the percentage incidence and mean found in Brazil (86 %/17 ng/L) **(Venâncio *et al.*, 2019)**, India (45.3 %/18 ng/L) **(Nile *et al.*, 2016)** and Ethiopia (26.3 %/) **(Gizachew *et al.*, 2016)**. While reports from China (74 %/100) **(Xiong *et al.*, 2018)** and Algeria (46 %; 72 ng/L) **(Mohammedi-Ameur *et al.*, 2020)** reported lower incidence but higher mean AFM_1 contamination in cow milk samples.

Table 1: Distribution of AFM_1 in raw animal milk and yoghurt in northern Nigeria.

Milk source	Total samples	Positive samples (%)	Samples above EU legal limit [a] (%)	AFM_1 concentration in positive samples (ng/L)		
				Min-max	Median	Mean ± SD
Cow	77	76(99)	32(41.6)	1-351	46.0	68.4±75.7
Camel	23	17(74)	5(21.7)	4-198	8.5	38.1±61.1
Goat	24	24(100)	20(83.3)	3-349	82.8	112±84.3
Yoghurt	20	20(100)	10(50)	5-537	49.0	145±163

Legend: [a]The EU legal limit of AFM_1 in milk is 50 ng/L (EC, 2006a). The values in parentheses indicate the percentages of samples.

At 95% confidence interval, no significant difference was observed in the incidence of AFM_1 contamination in cow milk samples collected from all states (Table 2).

Table 2: Occurrence of AFM_1 in raw cow milk from four states in Nigeria.

Location	Total samples	Positive samples (%)	Samples over EU legal limit [a] (%)	AFM_1 concentration in positive samples (ng/L)		
				Min-max	Med.	Mean ± SD
Bauchi	7	7(100)	0	69-116	93	$92.0±15.6^{b}$
Kaduna	24	24(100)	22(91.7)	28-351	123	$143±82.7^{a}$
Katsina	23	23(100)	2(4.4)	4-161	13	$18.4±31.6^{bc}$
Plateau	23	22(96)	8(34.8)	1-118	12	$33.4±38.2^{bc}$

Legend: [a]The EU legal limit of AFM1 in milk is 50 ng/L (EC, 2006a). The values in parentheses indicate the percentages of samples. Superscript letters indicate significant difference ($p < 0.05$) compared with the cow milk samples from other state as assessed by ANOVA. Med. - Median

However, cow milk from Kaduna state contained significantly ($p < 0.05$) higher levels of AFM_1 with 92 % of the samples collected exceeding regulatory limits and maximum levels as high as 351 ng/L. In goat milk samples, the incidence and mean concentration of AFM_1 in this study is akin to previous reports on AFM1 in similar samples from South Africa (100 %/62 ng/L) **(Mulunda, 2016)**. Conversely, lower incidence and mean concentration of AFM_1 was reported in goat milk samples from India (33.3 %/14 ng/L) and camel milk from Iran (4 %/9 ng/L) **(Fallah *et al.*, 2016; Nile *et al.*, 2016)**. In the present study, 22 %, 42 % and 83 % of camel, cow and goat milk samples, respectively, exceeded the EU MAL of 50 ng/L, with the concentrations reaching 198, 351 and 349 ng/L correspondingly.

The incidence (max level) of AFM_1 in *kindirmo* sample in this study is in agreement with the 100 % (700 ng/L) incidence reported by **Okeke *et al.* (2012)**. Our result, however, contradicts the finding by **Okeke *et al.* (2012)** who reported concentration of AFM_1 above the EU acceptable limit in all 10 samples analyzed using High Performance Liquid Chromatography as against the 50 % of samples above safe limits in this study. The difference in AFM_1 levels within EU acceptable limits reported in *kindirmo* samples in the present study and the study by **Okeke *et al.* (2012)** may have been influenced by a number of factors such as sample size, dairy animal breed, fermentation technique and particularly analytical method applied since the previous report applied a more sensitive technique.

Our findings on AFM_1 contamination of raw animal milk and milk products adds to previous data from Nigeria (**Makun *et al.*, 2010; Okeke *et al.*, 2012; Oluwafemi *et al.*, 2014; Oluwafemi and Lawal, 2015; Susan *et al.*, 2012)** and other countries **(Xiong *et al.*, 2018; Venâncio *et al.*, 2019; Mohammedi-Ameur, *et al.*, 2020)**. To the best of our knowledge, we report for the first time the presence of AFM_1 in camel milk and goat milk in Nigeria albeit there are reports on the consumption of camel and goat milk in the country.

Limitations of the study and potential health impacts of AFM_1 in animal milk and milk products

This study was limited by the application of ELISA, which has known limitations such as cross-reactivity leading to less sensitivity compared to high-end methods **(Oplatowska-Stachowiak *et al*., 2016)**. However, ELISA remains an important tool for screening purposes especially in developing countries, where there is limited access to high-end analytical tools **(Makinde *et al*., 2020)**. Notwithstanding, a liquid chromatography tandem mass spectrometric-based method is necessary to accurately quantify the levels of AFM_1 and other potential co-occurring mycotoxins in the milk samples. Considering its status as a class 2B carcinogen **(IARC, 1993)**, the high prevalence of AFM_1 in the animal milk and *kindirmo* samples raises serious food safety concerns. Widespread consumption of these dairy products by households including by infants and young children has been documented in Nigeria **(Nnadozie *et al*., 2014; Midau *et al*., 2010a; Midau *et al*., 2010b)**. Thus, there are indications that consumption of animal milk and its locally fermented products such as *kindirmo* could be a contributing factor to aflatoxin exposure recorded in low-income households in northern Nigeria.

CONCLUSION

This study suggests that raw animal milk and dairy products available for consumption in northern Nigeria are contaminated by AFM_1 and at concentrations that exceed regulatory limits. As regulation does not cover locally produced raw animal milk, there is a need for massive education and awareness on the importance of mycotoxins and useful approaches for limiting aflatoxin contamination of animal feed, which is the primary source of AFM_1 in milk and dairy products. In addition, strict monitoring of the local milk production chain is required. Since AFM_1 is relatively stable to high and low temperatures used for sterilization and storage respectively, we therefore recommend that optimal animal feed production and storage measures should be adopted to reduce aflatoxin contamination in milk.

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Declaration of interest

Authors do not have any interests to declare regarding this work.

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