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REGULAR ARTICLE

POTENTIAL IN BIOETHANOL PRODUCTION FROM VARIOUS AGRO WASTES FERMENTING BY MICROORGANISMS USING CARROT PEEL, ONION PEEL, POTATO PEEL AND SUGAR BEET PEEL AS SUBSTRATES

Isaie Mushimiyimana[1]*, *Fidel Niyitanga*[2], *Celestin Sirimu*[3]

Address (es):
[1,3]Department of Food Science and Technology, College of Agriculture, Animal Science and Veterinary Medicine, University of Rwanda (CAVM /UR)
[2]Department of Rural Development and Agricultural Economics College of Agriculture, Animal Science and Veterinary Medicine, University of Rwanda (CAVM/UR)

*Corresponding author : muzayasi@gmail.com

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ABSTRACT

Large amount of agro wastes are produced in Rwanda each year. The global annual potential bioethanol production from the major vegetables wastes such as carrot peel, onion peel, potato peel and sugar beet peel was estimated. Those wastes processing were successfully used as raw materials for the production of bioethanol, employing by cellulase produced from various filamentous fungi including *Cladosporium cladosporioides* was used for hydrolysis and the fermentation of the hydrolyzed samples was done using *Sacharomyces cerevisiae*. The fermented product was purified by primary distillation process at 79 °C and the fraction was collected. The ethanol is then determined by specific dichromate method and Gas Chromatography. Instantaneous saccharification and fermentation process yielded maximum ethanol in the substrate of carrot peel was 16.9 % at 21st day and further confirmed by Gas chromatography and the yield of ethanol obtained was 15.8 %.

Keywords: Agro wastes, Bioethanol, *Cladosporium cladosporioides*, *Saccharomyces cerevisiae*, Gas Chromatography

INTRODUCTION

During oil crisis in 1970s a rising crude oil price, political instability and unstable oil market in countries producing oil and climatic changes, biomass has high potential to replace the supply of energy **(Nagashima *et al.*, 1984).**The amount of wastes produced by society each day is increasing in line with increasing populations worldwide and Rwanda is no exception. Agro wastes are an important part of total solid wastes produced nationally; they have potential to become an environmental pollution or more logically, to be utilized for the production of energy and other products.

Production of bioethanol from lignocelluloses materials such as agro wastes can substitute fossil oil production. Today, raw materials producing bioethanol by fermentation are classified as sugars, starches and cellulosic materials because fermentation is cheaper and easily than other fermentation. The potential of bioethanol production from agro wastes of four crops which are carrot, onion, potato and sugar beet have been investigated. Currently, agro wastes are burnt by the rural farmers as cookers in households. Production of bioethanol from agro wastes have been attempted with enzymes from different sources for hydrolysis of biomasses and with different organisms for fermentation **(Ohgren *et al.*, 2006; Eken- Saracoglu and Arslan 2000)**.

The demand for bioethanol is expected to increase dramatically until 2020 where there is an increase in the world population with expected 9 billion in the year 2050 increasing the need for food and energy. *S. cerevesiae*, also known as brewer's yeast, is the most commonly used fermentation microbe because of the baking and beer brewing industries **(Michilka, 2007; Roehr, 2001)**. Many of the sugar crops that would be suitable for industrial fermentation include sugar cane, sugar beet, fruits, sweet potato, sweet sorghum, Jerusalem artichokes and agro wastes **(Atiyeh and Duvnjak, 2002; Pramanik, 2003)**.

The objective of this study was producing bioethanol from carrot peel, onion peel, potato peel and sugar beet peel for submerged fermentation and management system to maximize economic benefits at the same time protection of the environment.

MATERIALS AND METHODS

Raw materials

Carrot peels, onion peels, potato peels and sugar beet peels (Fig. 1), were collected from the local restaurant in volcanic region at early morning. They were clean to make free from sand, stone and dust by washing it twice in water. They were sun dried then each raw material was grinded and sieved into a 1mm. Those agro wastes are favorable for bioethanol production due to their availability and cheapest throughout the year.

Figure 1 Raw materials: agro wastes

Microorganisms producing ethanol

Several bacteria, yeasts, and fungi have been used for bioethanol production. *S.cerevisiae*, is the most yeast, which can produce ethanol of the fermentation broth. The yeast *S. cerevisiae* can produce bioethanol up to 18% of the fermentation broth **(Pretorius, 2000)**.

Sources of microorganism

The isolated fungi were done from the rhizosphere of strawberry fields of College of Agriculture, Animal Science and Veterinary Medicine (Busogo) identified by serial dilution and wet mount technique **(Aneja, 2005)**.

Culture medium chemical

The fermentation used was 0.2% yeast extract, 0.2% (NH4) NO3, 0.1% MgSO4·7H2O, 0.2% KH2PO4 **(El-Gendy *et al.*, 2013)** and 5 g powdered of each substrate has been added.

Enzyme molecular weights
Poly-Acrylamide Gel Electrophoresis (PAGE) of the partial purified cellulase enzyme was performed according to **(Lemmli, 1970)**. After electrophoresis, the gel was immersed in fixing solution. Staining of the band was done with Coomassie brilliant blue, R-250 (CBB) for 2 h and later de-stained. The molecular weight of the cellulase was estimated using standard protein molecular weight marker consisting of Bovine Serum Albumin.

Protein estimation
The protein from partially purified samples of carrot peel, onion peel, potato peel and sugar beet peel were estimated **(Bradford,1976)** method. Optical density of the reaction mixture was observed at 660 nm against a blank prepared with 0.1 mL buffer.

Fermentation
Culture filtrate was further inoculated with *S. cerevisiae* and allowed for fermentation for 14th, 21st and 28th days. After fermentation, it was filtered and ethanol content was determined **(Caputi *et al.*, 1968).** As part of this study, we have reported a process for producing ethanol from agro wastes pre-hydrolysed by alkali followed by saccharification carried by co-cultivation of *C. cladosporioides* and fermentation of the released sugars to ethanol, using *S. cerevisiae* for ethanol production

Distillation process
Distillation was carried in rotary vacuum flask at 80 °C (boiling point of ethanol) and fraction is collected as shown on Fig 2.

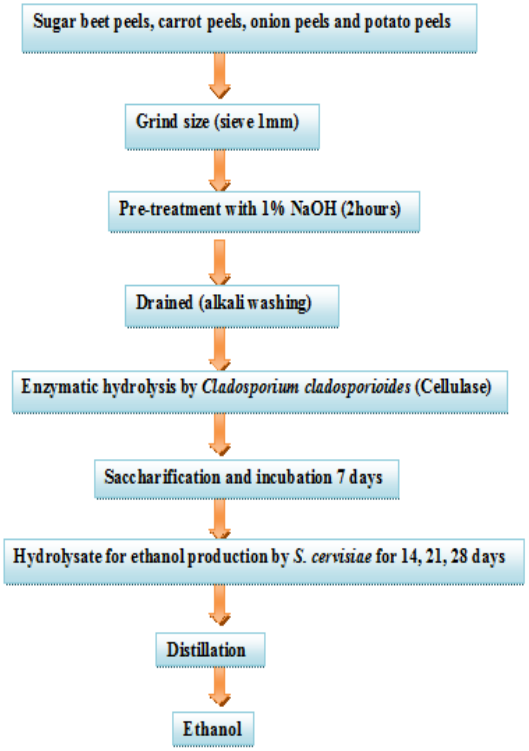

Figure 2 Bioethanol production from onion peel, sugar beet peel, carrot peel and potato peel

Bioethanol estimation by potassium dichromate method
Standard ethanol was prepared from concentrations of 2% to 10% with blank. 2.5 ml of freshly prepared potassium dichromate solution (1g of potassium dichromate in 100 ml of pre-chilled $_6H_2SO_4$) was mixed with 15ml of distillates and standards (2%, 4%, 6%, 8% and 10%) taken in separate test tubes and were incubated at 60 ºC for 30 minutes (for color appearance) **(Caputi *et al.*, 1968)**. Tubes were allowed to cool to room temperature and absorbance was estimated at 600 nm **(William and Reese, 1950)**.

Determination of quantity of ethanol produced
The distillate collected was measured using a measuring cylinder and expressed as quantity of ethanol produced in g/l by multiplying the volume of the distillate by the density of ethanol (0.8033g/cm3).

Statistical Analysis
MS Excel version 2007 was employed for all statistical analysis. Data was recorded in triplicates and represented as a mean value.

RESULTS AND DISCUSSION

Currently bioethanol is produced from alcoholic fermentation of molasses or simple sugar, which are produced from crops generating starch or sugar. While technologies to produce ethanol from simple carbohydrates are well established, the technologies to produce bioethanol from agro wastes are still under development. It is possible that agro waste products may be economically converted to bioethanol. We used agro wastes peel as a source of lignocellulosic substrate for ethanol production (Figure1 and 2).

Enzyme molecular weights
The protein present in various agro wastes substrates showed several bands ranged from 30 to 130 kDa. The crude protein extract of carrot peel which contains maximum yield concentration of bioethanol confirmed its homogeneity and protein was resolved on 5% stacking and 12% running gel. The molecular weight of the protein bands were 30 kDa and 130 kDa for carrot peel (Figure 3).

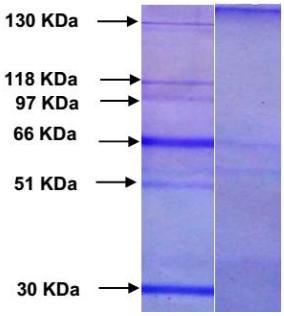

Figure 3 Molecular weight and cellulase activity from *Cladosporium cladosporioides*

Our results are close to the findings of **Bai *et al.* (2013)**. They reported that the the molecular weight of cellulase produced by different fungal species may vary from 12 kDa to 126 kDa. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly used method for judging the apparent molecular weight of enzymes **(Ramani *et al.*, 2012)**. Cellulase produced by *Trichoderma viride* was purified to homogeneity using DEAE-sepharose column and the molecular weight was estimated at 87 kDa by SDS-PAGE **(Yasmin *et al.*, 2013)**. *Penicillium pinophilum* MS 20 produced a monomeric cellulase with molecular weight of 42 kDa, which appeared as a single band on SDS-PAGE gel **(Laxman *et al.*, 2012)**. The cellulase produced by *Aspergillus niger* revealed a molecular weight of 60 kDa on SDS-PAGE gel **(Barlado *et al.*, 2014)**.

Protein estimation
The protein content with *C. cladosporioides* was observed in carrot peel 643.48 µg/ml, onion peel 1336.5 µg/ml, potato peel 1318.76 µg/ml and sugar beet peel 1101.12 µg/ml (Table 1). **Ado and Yabaya (2008)** reported the mycelial protein production by *Aspergillus niger* using banana peel. The protein content obtained by *Cladosporium* sp. with lignocellulosic biomass was about 0.224 mg/g and mycelial protein about 60.6±1.12 mg/g, reported by **Mohan *et al.* (2013)**.

Table 1 Substrates protein content (µg/ml) with *Cladosporium cladosporioides*

Carrot peel protein content (µg/ml)	Onion peel protein content (µg/ml)	Potato peel protein content µg/ml	Sugar beet peelprotein content µg/ml
643.48 µg/ml	1336.5 µg/ml	1318.76 µg/ml	1101.12 µg/ml

Mango peels, ranged from 1.2258–13.8715 mg/ml in which *Aspergillus tamarii* produced the maximum protein concentration released on day 12 of cultivation. Watermelon peels, it ranged from1.8926–5.2474 mg/ml in which *Aspergillus terreus* gave the maximum biosynthesis potential on day 3 of fermentation. The yield of extracellular protein on the rampage on medium containing banana peels ranged from 0.9247-4.0108 mg/ml in which *Mucor piriformis* had the maximum biosynthesis potential on day 3 of submerged cultivation. Furthermore, on medium with plantain peels, it ranged from 1.1725-8.3441 mg/ml in which *Aspergillus sclerotioniger* had the maximum biosynthesis potential on day 3 of

cultivation. *Aspergillus* sp. take over *Fusarium* sp. and *Mucor* sp. in polygalacturonase (PG) production.

Bioethanol obtained by dichromate method

Lignocellulosic materials and various agro wastes with different methods have been employed for bioethanol production. The maximum level of bioethanol varied from day to day fermentation. During the fermentation period, the ethanol yield of substrates was found to increase gradually from the 14th, 21st to 28th day (Figure 4). The maximum concentration of ethanol was achieved on 28th day of fermentation and started to level off. From the results obtained on bioethanol production potential of various lignocellulosic wastes varied and can be concluded that carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides*. **Mishra *et al*. (2012)** founded increase in quantity of ethanol produced in sub-merged state fermentation as compared to the produced by solid state fermentation and founded optimal incubation period 72 hours for bioethanol production by orange peel using *S. cerevisiae*. **Senthilkumar and Gunasekaran (2005)** reported that some Gram-positive bacteria *Clostridium cellulolyticum*, *Lactobacillus casei* have been engineered for bioethanol production. **Dien et al. (2003)** worked on Gram-negative bacteria *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*. *E. coli* and *K. oxytoca* are naturally able to use a wide spectrum of sugars, and work has concentrated on engineering these strains to produce ethanol selectively.

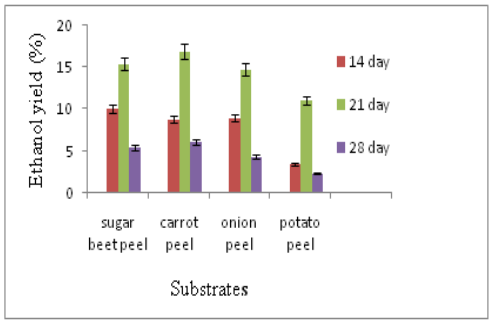

Figure 4 Ethanol obtained by dichromate method bioethanol yield (%) with agro wastes peel after 14th, 21stand 28th days of incubation of *Cladosporium cladosporioides*

Gas Chromatography and Bioethanol concentration

The Purity level for the ethanol through Gas Chromatography for the sample carrot peel was found to be to be 15.85 %, the Retention Time [min] was 1.06, and Area [mV.s] was 2400, Height [mV] 168.364 and Area [%] 93.4 (Figure 5).

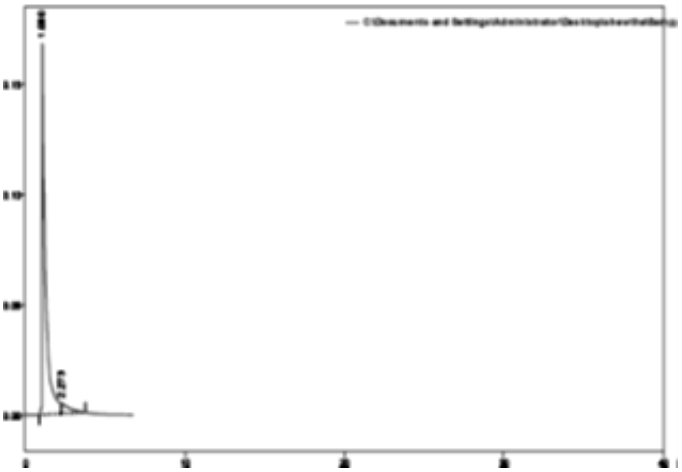

Figure 5 Gas Chromatogram of *Cladosporium cladosporioides* with carrot peel at 28 days**.**

Isaie and Padmavathi (2016) reported that agro wastes such as carrot peel, onion peel, potato peel and sugar beet peel are products subjected to saccharification process by *Penicillium* sp. for the hydrolysis, this process was followed by the fermentation using yeast *S. cerevisiae* for the production of alcohol which was fermented at 14, 21, 28 days to produce alcohol. High yield of ethanol was obtained from sugar beet peel 14.52 % on 28th day and further confirmed by Gas chromatography and the yield of ethanol obtained on 28th day was 17.3 %. **Muchtaridi *et al*. (2012)** determined alcohol contents of fermented black tape ketan based on different fermentation time with three different methods. Methods used are specific gravity, refractive index and GC-MS. Alcohol concentration obtained by using specific gravity method at 3, 10, 17, 24, and 31 days of fermentation, respectively, are 3.17 % v/v; 3.02 % v/v; 3.63 % v/v; 3 , 12 % v/v; and 4.47 % v/v, using the method of refractive index is 3.90 % v/v; 3.69 % v/v; 4.31 % v/v; 3.80 % v/v and 5.04 % v/v, and using GC-MS method was 4.30 % v/v; 4.23 % v/v; 5.01 % v/v; 4.75 % v/v; and 5.34 % v/v. The variation of fermentation time obviously did not influence the produce of alcohol contents statistically.

From the results obtained on bioethanol production potential of various lignocellulosic wastes varied, and can be concluded that carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides*. The maximum bioethanol concentration obtained in carrot peel at 21st day by *C. cladosporioides* was 133.341 g/l (Table 2).

Table 2 Bioethanol concentration by *Cladosporium cladosporioides* (g/l)

Substrates + *Cladosporium cladosporioides*	**Bioethanol yield (g/l) at 14th day**	**Bioethanol yield (g/l) at 21st day**	**Bioethanol yield (g/l) at 28thday**
Sugar beet peel	79.136	121.506	42.606
Carrot peel	69.353	133.341	47.734
Onion peel	7.101	115.983	34.400
Potato peel	27.615	87.026	18.620

From the results obtained on bioethanol production potential of various lignocellulosic wastes varied, and can be concluded that carrot peel was a very promising raw material for bioethanol production with *C. cladosporioides*. The maximum bioethanol concentration obtained in carrot peel at 21st day by *C. cladosporioides* was 133.341 g/l (Table 2). **Oyeleke *et al*. (2009)** reported that the maximum volume of ethanol (27.10 g/l) produced from guinea corn husk and millet husk (18.24 g/l) at the 120th hours with *Zymomonas mobilis*. **Agulejika *et al*. (2005)** reported maximum ethanol yield at 120th hour from fresh fruit (64.01 g/l) and waste fruits (21.14 g/l) using *Zymomonas mobilis*. **Ismail *et al*. (2012)** has reported yields of bioethanol 0.475 g/g to 0.51 g/g of the Wheat Straw and corn cobs, and hulls acid hydrolysate respectively. Using green algae **(Trivedi *et al*., 2013; Ge *et al*., 2011)**. **Wu *et al*. (2014)** obtained an ethanol yield of 0.45 g/g from *U. fasciata*, 0.44 g/g from *Laminaria japonica* and of 0.47 g/g from hydrolysate *Gracilaria* sp..

CONCLUSION

The present study examined the influences of fermentation period on ethanol production ability of *S. cerevisiae* using the carrot peel, onion peel, potato peel and sugar beet peel as substrates. The results of this study indicate incubation time for fermentation using *S. cerevisiae* which may enhance ethanol yield and minimize the cost of production could be obtained from agro wastes as substrates. Bioethanol production by *S. cerevisiae* may be used as successful alternative of *S. cerevisiae* in bioethanol production.

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Conflict of Interest Statement: The authors declare that there is no conflict of interest.

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REGULAR ARTICLE

ISOLATION AND MORPHOLOGICALLY IDENTIFICATION OF *ASPERGILLUS FLAVUS* INCIDENCES FROM MAIZE SEEDS IN ABUJA, NIGERIA

Hajara Oyiza, Yusuf[*1], *Joshua Olu*[2], *A. J. Alu*[3], *T.S. Anjorin*[4]

Address (es):
[1]Environmental Biotechnology and Bio conservation Department, National Biotechnology Development Agency, Along Musa Yar'adua Expressway, Airport Road, Lugbe –Abuja, PMB 5118, Wuse Zone 3, Abuja.
[2]Centre for Environmental Studies and Sustainable Development, Lagos State University, Ojo.
[3]Crop Science Laboratory, Faculty of Agriculture, University of Abuja.
[4]Department of Crop Protection, Faculty of Agriculture.

*Corresponding author : haj_yusuf@yahoo.ca

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ABSTRACT

Food safety and security well implemented could help in making more crops available for consumption. Maize seed is a crop well known to be attacked by fungi such as *Aspergillus flavus* and reduce its nutrients. This study intends to isolate and morphologically identify the *Aspergillus flavus* from maize seeds from Abuja, Nigeria. The experimental design was complete randomized design involving untreated yellow (Y) and white (W) maize seeds from7 locations in Abuja, Nigeria. Pure culture of fungal isolate was prepared using Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA). Isolates obtained were characterized and identified on the basis of their colonial and morphological characteristics which include macroscopic and microscopic examinations. All maize seeds from the Abaji had no fungi incidences in both SDA and PDA, while all maize seeds from the experimental field show fungi incidences in both SDA and PDA. On SDA, the maize color yellow and white had F (2, 6) static values of 7.083 and 0.212 at p=0.129 and 0.941 respectively. For the PDA, white maize seeds and yellow maize seeds from all the locations had F (2, 6) static values of 0.377 and 0.521 at p=0.850 and 0.773. *Aspergillus flavus* was isolated in this study. The maize seeds from Kuje district and the experimental field 2 show a high incidence records. All growth of the *Aspergillus* in the two media used were not significant at p>0.05.

Keywords: Maize seed, *Aspergillus flavus*, food safety, fungal incidences, Aflatoxin

INTRODUCTION

Fungi are known to cause deterioration and loss of nutrients in maize after insect (**Debnath *et al.*, 2012**). The fungi genera *Aspergillus, Bipolaris, Curvularia, Fusarium, and Penicillium* which are well known fungi that attack seeds have been linked with maize seeds (**Hussain *et al.*, 2013**). *Aspergillus flavus* is the most common member of the *Aspergillus* species in West African and the United States soils (**Gachara *et al.*, 2018**). Systemically, fungal attack on maize replete its viability, nutrient quality and quantity, seedling blight, failure in germination, subdued seedling and unappreciable crop performance (**Enyiukwu and Ononuju, 2016**). *Aspergillus flavus* is a saprophytic pathogen that thrives largely on many organic nutrient sources with sugars (**Amaike and Keller, 2011**). It is a fungus with wide economic impact which cut across been a pathogen of animals and insects, plants, cause of storage rots in large number of crops, production of highly regulated mycotoxin, aflatoxin B1(**Klich, 2007**). Its aflatoxin contaminants had been reported in some agricultural products (**Perrone *et al.*, 2014**). *A. flavus* a well-known and cosmopolitan fungus could survive some series of environmental conditions (**Abbas *et al.*, 2009**). They have the tendency to survive temperatures within 12ºC to 48 ˚C, an optimal growth temperature of 28 ˚C to 37 ˚C and a high humidity above 80% **(Hell and Mutegi, 2011; Yu, 2012**). As a storage mold on plants products, Maize seeds have been reported to be infected by *A. flavus* in the field prior to their harvest and in storage **(Klueken *et al.*, 2009**).

Maize (*Zea mays* L.) a cereal crop belongs to the *Poaceae* family and it is rich in vitamins A, C and E, carbohydrate, protein, essential minerals, fibre and calories (**Salako *et al.*, 2019**). Millions of maize about 8.63 million Metric Tons (MT) is produced annually in Nigeria (**Sule *et al.*, 2014**). It is a staple for over 1.2 million individuals in Africa and Americas (**IITA, 2009; USDA, 2016**). Maize is used as raw material for some industrial production, feed, fodder, and vegetable. It has been reported that poor storage condition, storage period, temperature, humidity levels and suitable climate could lead to infection caused by various storage fungi, such as *Aspergillus* species **(Ezekiel *et al.* 2008**)

This study intended to isolate and morphologically identify the *Aspergillus flavus* from maize seeds from Abuja, Nigeria.

MATERIALS AND METHODS

Study area

The maize seeds used in this study was obtained from seven (7) different locations across Abuja, Nigeria, Table 1 and Figure 1 illustrate their geographical location.

Table 1: Location where maize seeds were obtained with their geographical location

Maize seed source	Region in Abuja	Abbreviation	Latitude, Longitude
Bwari (BR) Market	Abuja North (ABN)	BR	9.3046N, 7.3768 E
Goza (GZ) Market	Abuja North (ABN)	GZ	8.9307 N,7.2994 E
Gwagalada(GL) Market	Abuja North (ABN)	GL	8.9308 N,7.0969 E
Experimental Field UNIABUJA	-	F	8.9807 N,7.1805 E
Kuje Market	Abuja South-ABS	KJ	8.8810 N,7.2281 E
Kwali Market	Abuja South-ABS	KL	8.8153 N,7.0363 E
Abaji Market	Abuja South-ABS	AB	8.5082 N,7.0348 E

Seed collections

Two different color of maize seeds were obtained from six different markets across Abuja and experimental field, University of Abuja (UNIABUJA). Distribution of the location where the maize seeds were obtained is as illustrated in Fig. 1.

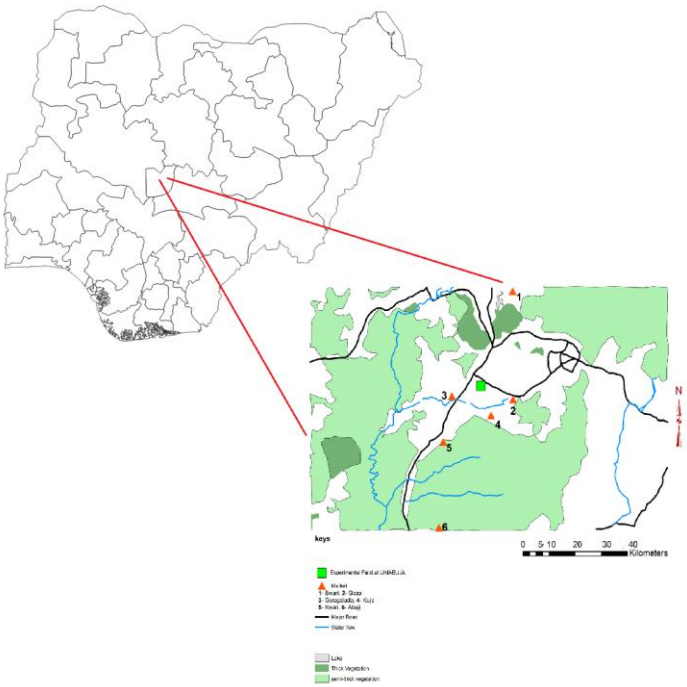


Figure 1: Map showing where the maize seeds were obtained in Abuja, Nigeria

Preparation and sterilization of media
Sabouraud Dextrose Agar (SDA)
SDA was used in this study and prepared according to the manufacturer's instructions thus, 65g of SDA is dissolved in 1000 ml of sterile water and then sterilized (autoclaved) at 121 °C and pressure of 15pa for 15 minutes

Potato Dextrose Agar
PDA was also used in this study and prepared according to the manufacturer's instructions thus; 39g of PDA powder was added to 1 liter of distilled water and boil while mixing to dissolve completely. The sterilization was done at 121ºC for 15 minutes using the autoclave. The sterilized prepared media was dispensed aseptically into petri dishes.

Preparation of pure culture of fungal isolate
The young fungal colony were aseptically picked up and transferred to fresh sterile SDA and PDA plates to obtain pure culture. The pure cultures on SDA and PDA plates were grown at 25 ± 2 °C for 7days and kept under 4 °C in a refrigerator. The isolates were subculture to obtain young cultures for further studies (**Klich, 2000**).

Identification of the fungal isolate
Cultural identification
Twelve isolates obtained from subculture were characterized and identified on the basis of their colonial and morphological characteristics which include macroscopic and microscopic examinations. Among the characteristics used were colonial characteristics such as size, surface, appearance, texture, and reversed pigmentation of the colonies of sporing structures. Appropriate references were done by using mycological identification keys and taxonomic description (**Harrigan and McCance, 2006**).

Morphological Characterization of *Aspergillus flavus*
Morphological attributes as described by **Klich (2002) and Clayton in Thathana *et al*., (2017**) were then utilized for further verification the isolates. Attributes such as colony color, colony growth, colony texture exudation which could be classified as macroscopic characteristics were studied. For microscopic analysis, attributes such as vesicles, asconidiophores, phialides, matulae and conidia were observed under the microscopic analysis of the isolate. Riddell's classic slide culture method **(Thathana *et al*., 2017**) and a method described by **Diba *et al*., (2007**) were used for the cultivation of the isolation the microscopic slides. Motic BA210 Basic Biological Light Microscope (Motic Instruments Inc., Richmond, BC, Canada) were used to examine the prepared slides using the immersion oil (100x) objective lens.
Incidence of fungi
Incidences of fungal infection on each sample were calculated by using the following formula:

In (%) = (Number of infected seeds) / (Total number of seeds) ×100.

RESULTS AND DISCUSSION

The incidence of the fungi was calculated and stated as indicated in Table 1. In this study, from findings stated in Table 2, there was more fungi incidences with the potato dextrose agar (PDA) compared to the Sabouraud Dextrose Agar (SDA).

Table 2: Mean Incidence of Fungi on yellow and white maize seeds collected from field and farmer store across Abuja

No.	Sample code	Incidence %	
		SDA	PDA
1	FY	14	11.23
2	FW	2.9	6.3
3	GLW	0.0	0.0
4	GL Y	10.2	6.5
5	BR W	0.0	0.0
6	BR Y	0.0	8.0
7	GZ W	0.0	16.0
8	GZ Y	0.0	3.3
9	KJ W	0.0	0.0
10	KJ Y	30.4	14.4
11	AB W	0.0	0.0
12	AB Y	0.0	0.0
13	KL W	2.9	0.0
14	KL Y	5.8	0.0

Legend: F-Field, Y-yellow, W-white, GL- Gwagwalada, KL- Kwali, KJ- Kuje, BR –Bwari, GZ-Goza and AB-Abaji

All maize seeds from the Abaji (AB Y and AB W) had no fungi incidences in both SDA and PDA, while all maize seeds from the experimental field (F Y and F W) show fungi incidences in both SDA and PDA. The yellow maize seed overall show more fungi incidence than the white maize seeds. On SDA, from Table 3 the maize color yellow and white had F (2,6) static values of 7.083 and 0.212 at $p=0.129$ and 0.941 respectively.

Table 3: Analysis of variance of the maize types from the different location on the two media

Media type	Maize colour	F (2,6)	Significance
SDA	White	0.212	0.941
	Yellow	7.083	0.129
PDA	White	0.521	0.773
	Yellow	0.377	0.850

For the PDA, white maize seeds and yellow maize seeds from all the locations had F (2, 6) static values of 0.377 and 0.521 at p=0.850 and 0.773. The study by **Sowley *et* al., (2018**) also reported a non-significant fungal incidence occurrence from maize samples.

Phenotypic Characterization of the *Aspergillus flavus* Isolates

Macroscopic Characteristics of the Isolates on PDA
The colony characteristics of the isolates are shown in Fig. 2 at the inception, the isolates were seen to have mycelia white color.

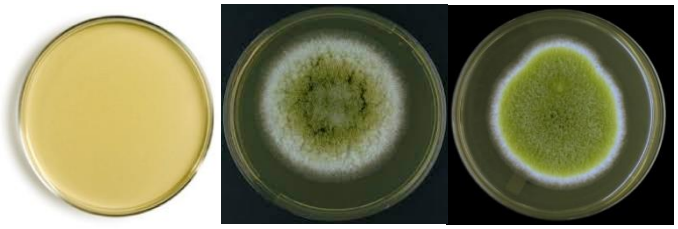
Control *Aspergillus flavus*
Figure 2 : Colony morphology of *Aspergillus flavus* isolates on PDA

The isolates after three days were seen to produce olive and dark green conidia, which happen to be the predominant appearance of the colony. They look raised in the center but their edges appear to be flat and plain with wrinkled in pattern like a cerebri. Droplets of liquid that is brown or uncolored were produced by the isolates. Sclerotia that were deep brown in coloration were produced in the

isolates. The colonies were encircled by a white border, and the colony diameter ranged between 65 and 75 mm. The undersides of the colonies were slightly pale

Macroscopic Characteristics of the Isolates on SDA

The attributes of the isolated colony are shown in Fig. 3. On the SDA the isolate colony were at the inception white with a velvety soft surface. After four days of growth, a floccose was seen at the center with a raise.



Control *Aspergillus flavus*

Figure 3: Colony morphology of *Aspergillus flavus* isolates on SDA

Yellowish-green and olive conidia were produced by the colony during sporulation. The whole surface of the colony was covered by conidia the edges, where border whitish in color were seen. On the sixth day of incubation, the produced sclerotia which were white initially became deep brown. No droplet of liquid known as exudates was produced.

Microscopic Characteristics of the *A. flavus* Isolates

The isolates were examined to ascertain their definitive identification, the microscopic attributes (conidiophores, conidia, metulae, phialides and vesicles) (Fig. 4). The conidiophores appeared uncolored, thick walled, roughened and vesicles bearing. Their diameter ranged between 800 and 1200 µm. Some isolates exhibit vesicles that were subglobose and globose in others with difference in diameter, ranging between 1800 and 2000 µm. There were uniseriate or biseriate or both kind of cells. The phialides were situated on the metuale with the biseriate cells, but attached to the vesicle, in uniseriate cells. The vesicles were covered with the metulae and radiated in all directions from the vesicles. Globose with thin wall with 250 and 450 µm range in diameter made up the conidia.

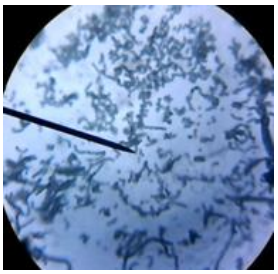


Figure 4: *Aspergillus flavus* sporex100

According to **Da Gloria (2011)**, both field and storage fungus contamination incidences in maize may vary among farms or producers in the same regions. This study share this view, for instance all the maize from Abaji-AB (yellow and white) show no fungal incidence in both PDA and SDA medium as illustrated in Table 2. While the case of Gwagalada-GL and Kuje-KJ yellow side there was fungal incidence in both PDA and SDA. The environmental situations and conditions could be a major determinant in the varying occurrences of *A. flavus* in the various districts of Abuja indicated in this study. Warm climate play a significant role in a huge chance of infection by aflatoxin producing fungal in some regions and this infection occurs only when there is drought with increase in temperature (**Cotty and Jaime-Garcia, 2007**).

Diba *et al*., (2007) examined the morphological characteristics of *Aspergillus* species from some specimens and they indicated that *Aspergillus* growth and conidia production maybe fasten if potato dextrose, malt extract, or likewise sporulation agars are used in growing them. This study used potato dextrose agar which from Table 2 show more incidence than SDA. Morphological methods as used in this study, had been reported to be used in identifying Aspergillus species from the soils in Fars and Kerman provinces of Iran (**Mohammadi *et al*., 2009**) and Larkana district in Pakistan (**Afzal *et al*., 2013**).

CONCLUSION

Aspergillus flavus was isolated in this study and the maize seeds from Abuja South Kuje district and the experimental field shows a high incidence records. All growth of the *Aspergillus* in the media were not significant at $p>0.05$. The maize seeds that recorded more incidences of *Aspergillus flavus* could be infected by higher levels of aflatoxins that cause some ill-health issue to humans, animals and plants. It will be wise then for fungus to be fight to its minimum in crops.

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REGULAR ARTICLE

INCIDENCE OF MYCOTOXINS IN MOULDY SMOKED DRIED FISH AND MEAT (KUNDI) MARKETED IN SOUTHWESTERN NIGERIA

Fatima O. Wambai[1], Olusola Awotula[1], Oluwapelumi A. Oyedele[2], Chibundu N. Ezekiel[2], Adeyinka O. Akintunde[1], Grace O. Tayo[1]*

Address (es):
[1]Department of Agriculture, Babcock University, Ilishan Remo, Ogun State, Nigeria.
[2]Department of Microbiology, Babcock University, Ilishan Remo, Ogun State, Nigeria.

*Corresponding author: tayog@babcock.edu.ng

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ABSTRACT

The mycotoxicological safety of 85 mouldy dried fish (n=40) and meat (n=45) samples purchased from Ijebu-ode, Ogun State and Aleshinloye, Ibadan, Oyo State markets both in South Western part of Nigeria respectively, was assessed due to heavy consumption of these animal protein sources by many low-income families in Nigeria. The presence of aflatoxigenic moulds and levels of aflatoxins in the samples were determined by the dilution plating technique and high performance thin-layer chromatographic method. The predominant fungal species isolated from the samples was *Aspergillus niger*-clade (49.65%); other species isolated include *Aspergillus* section *Flavi* (36.83%) and (13.52%). All samples analysed for mycotoxin presence were contaminated with aflatoxin B_1. Aflatoxin B_2, G_1 and G_2 were below detectable limits. About 13% of the samples had aflatoxin B_1 concentration higher than the maximum acceptable level (10 ng/g). Results imply that the consumption of mouldy dried meat can result in serious public health hazard and hence there is need for advocacy programs to enlighten the populace on proper processing and storage of meat products.

Keywords: Aflatoxin, Contamination, Food safety, Fungal

INTRODUCTION

Food safety and security remain a major concern in the sub-saharan Africa **(Bankole and Adebanjo, 2003)**, several food contaminants threaten the integrity of food products and make food unfit for consumption. Amongst the several food contaminants, mycotoxin in food commodities remain a challenge. In Nigeria, meat and fish consumption is usually associated with individual's socio-economic status, meat has been processed and eaten in various form that include *suya*, *kilishi* and *tinko* (**Obanu *et al*., 1987**). Dried meat also known as *kundi*, *tinko* and *banda* in Yoruba and Hausa respectively is a cheap, poor quality meat derived from various types of livestock (donkeys, horses, camel and buffalo) (**Adeyeye, 2016**). It is usually salted and spiced with condiments like ginger and garlic before being subjected to drying. When drying by sun or wind, the potential source of polycyclic aromatic hydrocarbons is the environment (**Ekhator et al, 2018**). Contamination can originate from soil/dust or/and from combustion from industry and from traffic as well as forest fires and volcanic eruptions (**Manisalidis et al, 2020**). This form of processed meat is included in diet because meat is a nutritious food that contains quantities of essential amino acids in forms of protein, it contains B group vitamins especially niacins and riboflavin, iron, phosphorus, ash and calcium. According to **Omotosho (2004)**, the recommended daily minimum protein required by an adult in Nigeria is between 65 and 85g per person and 35g of this minimum requirement should be obtained from animal products.

Chitrakar et al. (2019) reported that dried food including fish and meat are not inherently safe because of food borne illnesses caused by consumption of dried foods contaminated with *Salmonella* spp., *Cronobacter* spp., *Staphylococcus* spp. and *E. coli*. **Adesokan et al. (2016)** opined that the major source of contaminants especially aflatoxins was as a result of poor handling, packaging and storage of fish and meat alike. The processing technique involved in the processing of Kundi and dried fish; storage conditions and transportation mode predispose the meat product to mould growth and mycotoxin production. Mycotoxins are secondary metabolites produced by fungi in various food commodities and feed (**Adeyeye, 2016; Ezekiel et al., 2012; Atanda *et al*., 2013**). Out of the 300 secondary metabolites produced by fungi, about 30 metabolites have been found to be harmful and toxic among which Aflatoxins are a group of mycotoxins produced by *Aspergillus* subgenus section *Flavi* particularly *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergilus cerealis* (**Ezekiel *et al*., 2014**). Aflatoxins are grouped into namely AFB_1, AFB_2, AFG_1, AFG_2. AFB_1 is considered to be the most potent metabolite, it has been classified under class 1 human carcinogen by International Agency for Research on Cancer (IARC), it is highly hepatoxic, mutagenic and genotoxic (**Seo et al., 2011; IARC. 2012**).

Considering the detrimental effects of aflatoxins in the human system, there is need to assess and quantify the metabolites in staple and essential food products to ensure food safety. This study therefore aimed at quantifying the level of aflatoxins in dried fish and meat sold in Ogun and Oyo states of Nigeria.

MATERIALS AND METHODS

Sampling

Samples of smoked dried fish (n = 40) and smoked dried meat (n = 45) were purchased from vendors in markets in Ogun and Oyo states of Nigeria, respectively. The markets in both states were purposively selected because they are major depot for both commodities. Only vendors who belonged to the commodity association and had at least 1–2 kg food sample in store were selected. A total of 10–15 vendors per food category were selected among those who met the aforementioned criteria for sample collection. Each vendor was interviewed in order to retrieve information on the source, storage conditions and duration as well as extent of consumption of the foodstuffs.

Each sample (approximately 100 g) comprising of 5–10 visibly moldy pieces of either smoked dried fish or meat were collected by simple randomization from a vendor's tray/basket in the open market. Fish samples were purchased once every two weeks for two months while meat samples were obtained once every month for three months. Each sample was comminuted, quartered to yield about 25 g sub-sample and kept at -20ºC until further analysis.

Mycological analysis of food samples

Isolation of moulds

The dilution plating technique of **Samson *et al*. (1995)** was employed to assess the mycological profile of the dried fish and meat samples. Briefly, 10g of the ground samples were suspended in 90ml of sterile distilled water, homogenized for 2 minutes and spread-plated on potato dextrose agar (PDA) supplemented with 0.02% chloramphenicol and streptomycin. All isolations were performed in triplicates and the inoculated plates were incubated for 3–5 days at 31ºC. All colonies were counted and fungal load expressed as Log_{10} colony forming units per gram (log_{10}CFU/g) of sample analyzed. Colonies of *Aspergillus* were purified on PDA and transferred onto neutral red desiccated coconut agar (NRDCA) for further characterization as described by **Ezekiel *et al*. (2014)**.

Characterization of isolated moulds

The isolates on NRDCA were examined for their taxonomic confirmation. The species of *Aspergillus* was identified by assessing the morphology and for mycotoxin characterization, the aflatoxin-producing potential was determined. After five days of incubating the inoculated NRDCA plates at 31ºC, the macro

characters of species were assessed and compared with taxonomic descriptions in literature **(Samson *et al.*, 2002; Şesan *et al.*, 2007)**. To determine the isolates that produce aflatoxins, each isolate was centrally inoculated on freshly prepared plates and incubated in the dark at 30°C for 3 to 5 days. Each plate was checked under UV light (365nm wavelength) at 24 hours interval for fluorescence resulting from aflatoxin liberation and color fluorescence to determine the type of aflatoxin as previously described by **Ezekiel et al. (2014)**.

Aflatoxin determination in dried fish and meat samples

The fish and meat samples were analyzed for the presence of aflatoxins [aflatoxin B_1 (AFB_1), AFB_2, AFG_1 and AFG_2] by High Performance Thin Layer Chromatography (HPTLC) at the Pathology and Mycotoxin Laboratory of the International Institute of Tropical Agriculture, Ibadan, Nigeria. About 20 g of each sub-sample was extracted with 100 ml of 80% methanol by high-speed blending for 2 minutes and subsequent shaking for 30 minutes on an orbital shaker. The mixture passed through Whatman filter paper No. 1 and the filtrate was partitioned in a 250 ml separatory funnel to which 20 ml of 10% sodium chloride and 25 ml of hexane were pre-added. The methanol layer was collected into a 250 ml separatory funnel, mixed with 35 ml dichloromethane, shaken for 30 seconds and allowed to stand for separation. The lower dichloromethane layer was collected into a polypropylene cup and evaporated to dryness in a fume hood. The residue was re-dissolved in 1 ml of dichloromethane prior to aflatoxin quantification. Aflatoxin standards and sample extracts were separated on TLC plates (silica gel 60, 250 µm) in isopropanol-methanol-water (96:3:1, v/v/v). The plates were visualized under ultraviolet light and scored visually for the presence or absence of aflatoxin with a 2 ng/g limit of detection. Quantification was performed by the scanning densitometer as previously described (**Suhagia *et al.*, 2006**).

Data analysis

The SPSS® v. 16.0 was used for data analysis. Simple descriptive statistics was performed for aflatoxin distribution.

RESULTS

In each of the three different months of sample collection as shown in Table 1, fifteen samples were obtained and analyzed for fungal contamination. In the first month, thirteen of the samples were contaminated. In the second month, six of the samples were contaminated. In the third month, all fifteen samples were contaminated. Three specie of fungi were observed from the isolates.

Table 1: Frequency and Percentage of Fungal Isolates

		FREQUENCY	PERCENTAGE (%)
MONTH 1	*Aspergillus* section *Flavi*	48	48.98
	Aspergillus niger – *clade*	30	30.61
	Rhizopus spp.	20	20.41
MONTH 2	*Aspergillus* section *Flavi*	37	56.92
	Aspergillus niger – *clade*	25	38.46
	Rhizopus spp.	3	4.62
MONTH 3	*Aspergillus* section *Flavi*	73	27.44
	Aspergillus niger – *clade*	158	40
	Rhizopus spp.	35	13.16

Tables 2 and 3 show the range of Aflatoxin B_1 found in both dried meat and fish obtained in the markets. The results showed that the overall mean of AFB_1 detected in the meat samples and fish samples were more than 4µg/kg and 2µg/kg respectively.

Table 2: Distribution of Aflatoxin B_1 in Fish Sample

			Aflatoxin B_1(ng/g)	Concentration
Weeks	**Ns**	**Nc**	**Range**	**$\bar{X}$ ± SD (ng/g)**
1	10	2	0.00-8.11	2.92±3.85
2	10	4	5.05-7.94	5.97±1.33
3	10	2	0.00-5.93	2.75±3.19
4	10	3	4.87-6.59	5.71±2.88
Total	**40**	**11**	**0.00.8.11**	**4.25±2.88**

Legend: Ns – number of samples obtained, Nc –number of samples contaminated, $\bar{X}$ – mean, SD – standard deviation

Table 3: Occurrence of Aflatoxin B_1 in Dried Meat

				Aflatoxin B_1 concentration (ng/g)		
	Ns	**Na**	**Nc**	**RANGE**	**$\bar{X}$**	**S.D**
MONTH 1	15	5	5	3.10 – 6.93	4.66	1.52
MONTH 2	15	5	5	1.92 – 5.09	3.49	1.24
MONTH 3	15	5	5	2.60 – 18.94	8.53	6.82
ALL SAMPLES	45	15	15	1.92 – 18.94	5.56	4.40

Legend: Ns – number of samples obtained, Na – number of samples analyzed, Nc –number of samples contaminated, $\bar{X}$ – mean, SD – standard deviation

DISCUSSION

The results showed that the overall mean of AFB_1 detected in the meat samples and fish samples exceeded the stringent regulation of 4 µg/kg and 2 µg/kg respectively, set by the EU and this poses a great threat to the consumer's health because AFB_1 is a potent human carcinogen **(IARC, 2002)** that has been linked to hepatocellular carcinoma.

This was also in agreement with the findings of **Adesokan *et al*. (2016)** who investigated the aflatoxin concentration of smoked dried fish collected from Ibadan. In contrast to the aflatoxin levels in smoked dried fish sold in different markets in Abeokuta by **Akinyemi *et al*. (2011)**, the AFB_1 detected in the fish samples exceeded the results obtained from the samples collected from Abeokuta. Similarly, **Adebayo-Tayo *et al*. (2008)** reported high concentration of AFB_1 in smoked fish from Uyo, Nigeria. Human exposure by consumption of AFB_1 contaminated food can increase the chances of consumers developing hepatocellular carcinoma and stunted growth in children **(Turner *et al*., 2012)**. Considering the fact that dried fish and meat are highly consumed by the low-income populace of Nigeria, long term exposure can increase the occurrence of liver cancer. The findings from this study further substantiated the claims of **Chitrakar *et al*. (2019)** who concluded that dried food items especially fish and meat should be consumed with caution as they are not inherently safe.

CONCLUSION AND RECOMMENDATION

This study confirmed the presence of high concentration of AFB_1 in both dried meat and dried fish sold in the sampled markets. However, consumption of these food items as protein sources pose a great health hazard to consumers.

It is therefore recommended that regulation standards should be put in place and enforced to promote hygienic processing, transportation and storage of these animal products. There is also a need to educate both the traders and the consumers on the risk involved in consumption of such contaminated products

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REGULAR ARTICLE

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

*Muiz O. Akinyemi, Daniella O. Ekpakpale, Oluwawapelumi A. Oyedele, Kolawole I. Ayeni, Stephen O. Fapohunda, Chibundu N. Ezekiel**

Address (es):
Department of Microbiology, Babcock University, Ilishan Remo, Ogun State, Nigeria.

*Corresponding author chaugez@gmail.com

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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)**.

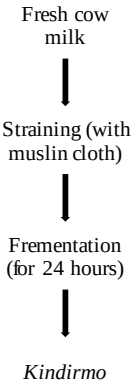

Figure 1: Flow chart for producing *Kindirmo*, a traditionally fermented yoghurt in Nigeria.

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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