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Evaluation of Aflatoxin-Producing Fungi in Indoor Air of Warehouses and Houses of Farmers in Giwa, Kaduna State Nigeria

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Abstract

The health risks associated with ingesting food contaminated with mycotoxins, particularly aflatoxin-contaminated staple foods like maize and other cereals, have been widely studied. However, there is little knowledge about the role of inhalation of pathogenic fungi as bioaerosols in contaminated air from handling crops as an occupational health risk. This paper presents a study aimed at determining the level of airborne aflatoxin-producing fungi in the indoor air of grain stores in the Giwa community of Kaduna State. Indoor air was sampled using the settling plate technique from grain stores, warehouses and living rooms. Metrological data of the studied area were collected from the Institute of Agricultural Research, ABU, Zaria. Airborne mycofloral concentrations were determined, and colonies of *Aspergillus flavus* were identified. The isolates were screened for aflatoxin production on Neutral Red Desiccated Coconut Agar (NRDCA). Selected aflatoxin-producing fungal isolates were screened for the presence of aflD (nor-1), aflM (ver-1) and aflR genes by PCR. Sampling was done once every month from October to December 2020. Mycofloral concentrations were in the range of 2.77×10^3 – 4.05×10^3 and 1.55×10^3 – 2.17×10^3 CFU m^{-3} for grain stores and living rooms respectively. A total of twelve (12) strains of *A. flavus* were isolated from the indoor air of the grain stores and warehouses while none was obtained from the living room. Eleven (11) isolates were confirmed to be aflatoxigenic on NRDCA, presenting 30 CFU m^{-3} of the indoor air mycofloral composition. The aflD, aflM and aflR were amplified with aflD being the most detected gene from all the aflatoxin-producing mould isolates of *Aspergillus* species. The mycofloral concentrations in the grain stores were higher than those in the living room and, in all the sampling sites, exceeded the limit of the total mycofloral concentration of 500 CFU m^{-3} for agricultural and industrial environments. There were significant differences ($p < 0.05$) in the indoor mycofloral concentrations between the grain stores/warehouses and the living room. The presence of aflatoxigenic strains of *A. flavus* in the stores indicates that grain handlers and traders are at risk of occupational exposure to aflatoxigenic fungi and aflatoxins. Hence, they should wear protective materials for their safety while working in such stores.

Keywords: Aflatoxin-producing Fungi, Maize, Groundnut, Warehouse, Indoor Air, Aflatoxin-biosynthesis genes.

INTRODUCTION

Mycotoxins, which are frequently exposed to agricultural workers, can have serious negative health impacts on them (Mayer *et al.*, 2016). A well-known agricultural industry that involves the collection and trade of grains has a dusty environment where mycotoxins may be present (Halstensen *et al.* 2008). Grain handling is particularly important from the perspective of occupational health and safety due to the ongoing exposure to airborne moulds and their metabolites while performing such tasks. Additionally, epidemiological studies and experimental animal models have demonstrated that inhaling mycotoxins can have negative effects on human health that are

more severe than those caused by oral exposure (Amuzie *et al.*, 2008; Degen, 2011; Veigas *et al.*, 2018). Since they can be identified at the species level, fungi are frequently utilized as markers for mycotoxins (Halstensen, 2008). Infectious and harmful effects are associated with moulds and their metabolites respectively (Marin *et al.*, 2013; Benkerroum, 2020). Specific fungal genera, particularly *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps*, produce mycotoxins as metabolites (Agriopoulo *et al.*, 2020). They have a low molecular mass and are tiny, stable molecules (Bennett and Klich, 2003; Marin *et al.*, 2013). They have been discovered in peanuts, grains like corn, and other foods for both people and

animals (Mayer, 2016). The two common *Aspergillus* species linked to aflatoxin contamination are *Aspergillus flavus* and *Aspergillus parasiticus*. Crops in the field may become contaminated naturally or as a result of poor storage and/or processing conditions (Benkerroum, 2020).

There is limited knowledge of mycotoxins' incidence and exposure during grain handling, and the majorities of studies concentrate on elements of mycotoxin contamination of food and feed grains in relation to dietary intake (Veigas *et al.*, 2018). To our knowledge, no prior research has been done in the study area on occupational airborne exposure to mycotoxins during grain handling. Studying a renowned food grain growing/trading community like Giwa can provide information about the indoor air quality of grain stores and warehouses, insight into the safety of the stores while grain tradesmen are inside of them, and serve as a crucial guide that would enable appropriate action, guide, regulation, and policy to be taken by appropriate authorities.

This study aimed to identify aflatoxin-producing moulds in grain stores' indoor air and mycofloral concentrations as a potential occupational source of aflatoxin exposure. And also identify aflatoxin-producing genes from the identified isolates.

MATERIALS AND METHODS

Study Area

Giwa is a large food-crop growing community located on longitude 11.25 °N and latitude 7.47°E. The residents of Giwa Local Government Area are mainly farmers. The common grains cultivated by the populace include maize, beans, soya bean, groundnut and sorghum. Grain storage facilities include small stores, moderate, and big warehouses in which they keep their crop produce, large proportions of which are located in the market. They also keep some of the crop produce at home in a dedicated room.

Sample Collection

The samples were collected from warehouses in the market, stores at home, and living room. A total of thirty (30) samples were collected for the study. Sampling units were; maize store in residential building, groundnut store in residential building and living room. The sampling was done three times at an interval of one month on 22nd October, 19th November and 21st December 2020.

The Omeliasky gravimetric method of Awad and Mawla (2012) was used to sample the air. Replicates of Saboroud dextrose agar (SDA) plate of 8.5cm diameter incorporated with

chloramphenicol were exposed for 30min, at 1.5m above the floor (along the breathing zone) and 1m from the wall. Air samples were collected between 9.00 am to 12.00 noon following the method described by Anaya *et al.* (2019).

Meteorological data of the studied area over the studied period (October to December) was collected from Metrological Unit, Department of Soil Science, Institute of Agricultural Research, Ahmadu Bello University Zaria. The data include temperature (T), relative humidity (RH), wind speed (WNS), wind direction (WND) and rainfall.

Enumeration of Fungal Load from the Air Sample

Fungal colonies were counted at 4 days of incubation and fungal concentrations were determined by equation 1 (Awad and Mawla, 2012):

$$N=5a \times 10^4 (bt)^{-1}$$

where “N” is the fungal concentration (in), “a” number of colonies per Petri dish; “b” is the dish surface area (in); “t” is the exposure time (in minutes).

Identification of Moulds

The moulds were identified as *Aspergillus flavus* and *Aspergillus parasiticus* using lactophenol cotton blue stain as described by Hocking (2007). Briefly, a drop of lactophenol cotton blue stain was placed at the centre of a clean microscope slide. A fragment of the fungal colony with bright yellow-green/green and cream reverse appearance was removed from the colony edge using an inoculating needle. The picked portion was placed in the stained drop and gently teased. A cover slip was applied and examined under a microscope using a low and high-power objective lens.

Screening of the Isolates for Aflatoxin Production on Neutral Red Desiccated Coconut Agar (NRDCA)

Presumptive fungal isolates were screened for aflatoxin production ability using NRDCA as described by Atanda *et al.* (2011). Each isolate was inoculated on freshly prepared NRDCA, incubated at ambient temperature for 5 days, and examined. Isolates that produced yellow pigmentation on the reverse side of the plates and around the fungal colony as well as fluoresced under UV light (at 360nm) were regarded as aflatoxigenic moulds (Atanda *et al.*, 2011).

DNA Extraction and Amplification of Aflatoxin-Producing Genes

DNA extraction was done using the QIAamp DNA Mini Kit by Qiagen, using the procedure as described in their handbook (Qiagen, 2022).

Using a set of three primers (*aflR*, *ver-1* and *nor-1*), a multiplex PCR procedure of Criseo *et al.* (2001) as adopted by Davari *et al.* (2014) was used to detect genes involved in the aflatoxin biosynthesis. Five of the isolates of *A. flavus* and *A. parasiticus* (four aflatoxin-producing and the one non-aflatoxin-producing on NRDCA), were examined for the presence of three important aflatoxin genes (*aflR*, *ver-1* and *nor-1*), their critical information is presented in Table 1. The PCR reaction was performed in 25 μ L containing 2.5 μ L 1 X PCR

buffer, 0.75 μ L 50 mM MgCl₂, 0.5 μ L 10 mM dNTPs, 2 μ L of each primer, 0.2 μ L Taq DNA polymerase (1 U/ μ L), 5 μ L extracted DNA as a template and 8.05 μ L sterile distilled water. The overall reaction cycles were thirty-five (35): with heating at 94 °C for 5 min, continued by denaturation for 30 s at 94 °C, annealing for 30 s at 67 °C, elongation for 30 s at 72 °C, and a final extension of 10 min at 72 °C. The amplified product was electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized by UV illumination.

Table 1: PCR Primers Used to Amplify the Genes for Aflatoxin-Production (Criseo *et al.*, 2001)

Gene	Primer name	Sequence 5-3	Amplification size
<i>aflD</i>	<i>norF</i>	ACCGCTACGCCGGCACTCTCGGCAC	400bp
(<i>nor-1</i>)	<i>nor</i>	GTTGGCCGCCAGCTTCGACACTCCG	
<i>aflM</i>	<i>verF</i>	GCCGCAGGCCGCGGAGAAAGTGGTGGGGATATACTCCCGCGACACAGCC	600bp
(<i>ver-1</i>)	<i>verR</i>		
<i>aflR</i>	<i>aflrF</i>	TATCTCCCCCGGGCATCTCCCGGCCGTCAGACAGCCACTGGACACGG	1000bp
	<i>aflrR</i>		

Statistical Analysis

One-way ANOVA was used to compare the mean mycofloral count among the sampling locations using Science Analytical Software (SAS-JP Pro 14). Where significant difference was observed, Tukey's Honestly Significant Difference test was used to separate the significant differences among the means.

RESULTS

Description of Sampling Sites

The warehouses, an example of which is shown in Plate I, are typical of size 4m x 5m x 3m. They have small block holes near the roof

which serve as a source of ventilation. The grains are normally arranged along the walls on all sides closing on towards the door. A typical warehouse can be filled to more than 80% of the floor surface but is not stacked to the roof to allow for ventilation. The warehouses used were all built and put to use together. They have been in use for up to 30 years. The stores at home are typical of size 3m x 3m x 2.5m with similar ventilating holes as the market warehouses. They have been in use for a long time which is normally the age of the house. The duration of use is typically more than 30 years.



Plate I: Warehouse in the market; (a) exterior view and (b) interior view

Airborne Mycofloral Concentration in Grain Stores and Warehouses in Giwa

Tables 2 and 3 present mycofloral concentrations in the indoor air of maize and groundnut stores. These ranged from 2.77×10^3 to 4.06×10^3 CFU m⁻³. For the living room, it ranged from 1.55×10^3 – 2.17×10^3 CFU m⁻³. All (100%) of the air sampled from the grain stores

was found to be contaminated with moulds. The mycofloral concentration was above the permissible limit of 500 cfu for the total number of fungal particles set by the American Conference of Governmental Industrial Hygienists (ACGIH, 2011) and WHO (Shittu *et al.*, 2019).

Table 2: Concentration of Moulds in Indoor Air of Maize Store/ Warehouse in Giwa, Kaduna State

Mould Concentration (x 10 ⁻³ CFUm ⁻³)				
Month (2020)	Grain Store	Warehouse	Living room	p-value
October	2.77 ± 0.17 ^a	3.16 ± 0.21 ^a	2.17 ± 0.17 ^b	0.014
November	2.95 ± 0.08 ^a	3.25 ± 0.09 ^a	2.05 ± 0.08 ^b	0.002
December	3.10 ± 0.21 ^a	3.25 ± 0.17 ^a	1.55 ± 0.06 ^b	0.003

*Those with the same superscript across the row are not significantly different, according to Tukey's Honestly Significant Difference Test (p < 0.05).

*Climatic conditions on the day of sampling:

October: T °C (16-33), RH% (19-91), WNS km/h (3.0), WND ° (281)

November: T °C (15-30), RH% (23-61), WNS km/h (3.4), WND ° (281)

December: T °C (16-32), RH% (19-51), WNS km/h (2.4), WND ° (283)

Table 3: Concentration of Moulds in Indoor Air of Groundnut Store/ Warehouse in Giwa, Kaduna State

Mould Concentration (x 10 ⁻³ CFUm ⁻³)				
Month (2020)	Grain Store	Warehouse	Living room	p- value
October	3.00 ± 0.08 ^a	2.89 ± 0.17 ^a	2.17 ± 0.17 ^b	0.02
November	3.52 ± 0.13 ^a	4.06 ± 0.021 ^a	2.05 ± 0.08 ^b	0.002
December	3.79 ± 0.17 ^a	3.55 ± 0.08 ^a	1.55 ± 0.06 ^b	0.001

This table has the same footnotes as table 2

Aflatoxigenic Fungi Isolated from Grain Stores and Warehouses in Giwa

A total of twelve (12) aflatoxin-producing fungi were isolated from the indoor air of the grain stores, with one from each sampling unit at every sampling time. Eight (66.67%) isolates

were presumed to be *Aspergillus flavus* while four (33.33%) were *Aspergillus parasiticus*. The moulds were found to be present in all the sampling locations except for the living room (Table 4).

Table 4: Potential Aflatoxigenic Moulds Isolated from Grain Stores in Giwa

Mould	Grain Store		Warehouse		Living room	Total (%)
	Maize	Groundnut	Maize	Groundnut		
<i>A. flavus</i>	2	-	3	3	-	8(66.67)
<i>A. parasiticus</i>	1	3	-	-	-	4(33.33)
Total	3	3	3	3	-	12(100)

Key: - means no aflatoxin-producing mould was isolated

Aflatoxin-Production Ability of the Fungal Isolates

Eleven of the isolates produced aflatoxin (Table 5), and each showed Pastel blue fluorescence surrounding the mould colony on NRDCA under the UV lamp. Seven of these isolates were

found to fluoresce moderately while four were bright under UV lamp. Using equation 1, one colony of aflatoxin-producing fungi presents 30CFUm⁻³ of the mould composition in all the sampling sites.

Table 5: Aflatoxin Producing Potential of Moulds Isolated from Grain Stores in Giwa

Species	Isolate code	Fluorescence intensity
<i>Aspergillus flavus</i>	HM1	++
<i>Aspergillus parasiticus</i>	HG1	++
<i>Aspergillus flavus</i>	WM1	+++
<i>Aspergillus flavus</i>	WG1	-
<i>Aspergillus flavus</i>	HM2	++
<i>Aspergillus parasiticus</i>	HG2	+++
<i>Aspergillus flavus</i>	WM2	++
<i>Aspergillus flavus</i>	WG2	+++
<i>Aspergillus parasiticus</i>	HM3	+++
<i>Aspergillus parasiticus</i>	HG3	++
<i>Aspergillus flavus</i>	WM3	++
<i>Aspergillus flavus</i>	WG3	++

Key: +++: Bright fluorescence ++: Moderate fluorescence, -: no fluorescence (Atanda et al, 2011).

UJMR, Vol. 7 No. 2, December, 2022, pp. 79 - 86
PCR Amplicon(s) of *aflD*, *aflM* and *aflR* Genes from *Aspergillus* species Isolates Plate II shows amplified DNA fragments for *aflD* (*nor-1*), *aflM* (*ver-1*) and *aflR* genes (400bp, 600bp and 1000bp respectively) obtained from aflatoxigenic mould isolates. Only two of the

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 isolates had all three genes. One (that did not produce fluorescence) showed no amplicon of all the targeted genes. A comparison of the aflatoxigenic characterization of mould between culture and PCR is made in Table 6.

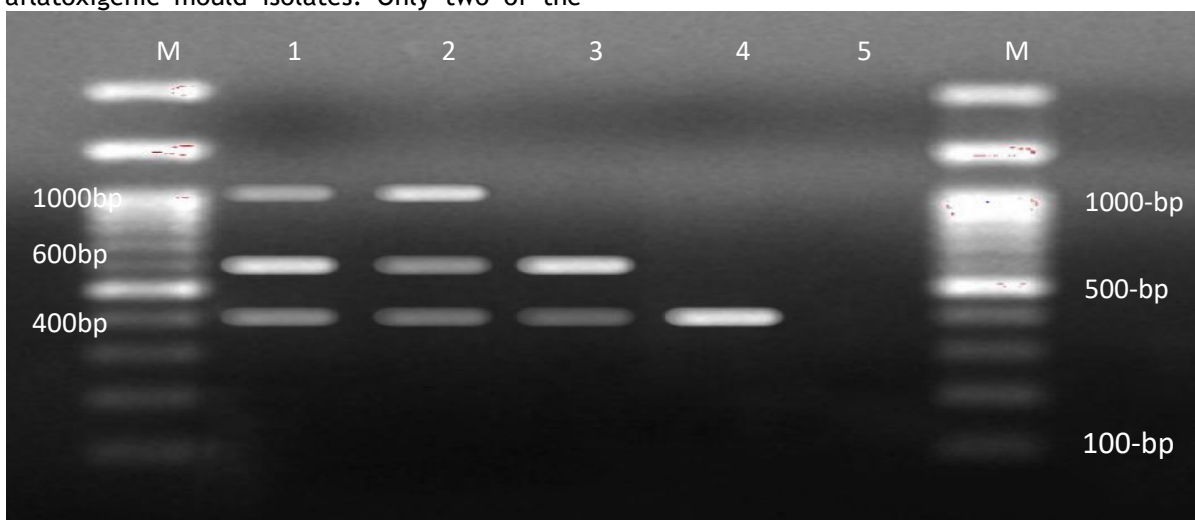


Plate II: Aflatoxin-production Genes Detected from some *Aspergillus* species Isolated from Indoor Air of Grain Stores

Key: M=Marker (100bp plus ladder, size 1500bp)

Lane 1: HM1, Lane 2:HG1, Lane 3:WM1, Lane 4:HM2, Lane 5:WG1.

Table 6: Aflatoxin Production and Detected Aflatoxin-Production Genes Using PCR

Isolate code	Mould species	Aflatoxin Production on NRDC	Aflatoxin-production gene		
			<i>aflR</i>	<i>aflM</i>	<i>aflD</i>
HM1	<i>Aspergillus flavus</i>	+	+	+	+
HG1	<i>Aspergillus parasiticus</i>	+	+	+	+
WM1	<i>Aspergillus flavus</i>	+	–	+	+
WG1	<i>Aspergillus flavus</i>	–	–	–	–
HM2	<i>Aspergillus flavus</i>	+	–	–	+

Key:

+: Fluorescence production/Gene detected

–: No fluorescence production/Gene not detected

DISCUSSION

The high mycofloral concentration in the stores and warehouses could be attributed to high wind speed and wind direction (see footnotes under Table 2) which have a significant influence on the indoor airflow (Aleksic *et al.*, 2017). The high concentration could also be attributed to the lack of proper ventilation in the stores and warehouses (as seen in the plate I). There were significant differences ($p < 0.05$) in airborne mycofloral concentrations among the sampling sites during all the sampling time. However, there was no significant difference in mycofloral concentration between stores and warehouses, but the significant difference was between stores/warehouses and the living room. These differences could be attributed to the aerosolized fungal spores/fragments from the stored grains and settled dust to the

ambient air which is not a characteristic of the living room (Mayer *et al.*, 2016).

In addition, during such grain handling as loading of grain sacks into the store, fungal particles are released from the surrounding layer of grain kernel and concentrate on the store's background, fungal particles settled on the store floor and grain sacks. Slight agitation (during grain handling and other trading practices) inside the store will increase the concentration of fungal spores and fragments (Mayer, 2016).

There was a slight increase in mycofloral concentration in the stores and warehouses from October to December in all the grain stores and warehouses, and this could be attributed to a significant decrease in relative humidity (90%, 61%, and 51% for October to December respectively) which might have

resulted to additional dryness of stored grains and ease release of microorganisms to the ambient air. Volenik *et al.* (2010) reported that the RH of the ambient air within a grain store influences the moisture content of the stored grains, also at RH below 65%, microorganisms do not grow and there will be no further contamination of the stored grains.

High mycofloral concentration in the indoor air of the living room in October and November could be attributed to the influence of outdoor air where some of the farmers in the community carry out harvesting processes. These include grain threshing and winnowing indoors or in a field within their residential neighbourhoods, thereby resulting in producing air rich in fungal particles.

As compared to the findings obtained, Lanier *et al.* (2010) reported higher levels of airborne moulds in agricultural environments (cattle farm where feeds are handled) of 1.4×10^3 – 8.9×10^4 CFU m^{-3} . The reason for the higher mycofloral concentration than that of this study could be due to the nature of the product handled in the studied environment which was feed, and it is known to contain a large amount of mycotoxins when compared to food (Udomkun *et al.*, 2017). Mycofloral concentration of 1.4×10^4 – 1.20×10^5 CFU m^{-3} was reported by Adhikari *et al.* (2004) in a farmland with highest concentrations during such tasks as grain unloading and handling. In this study, a higher concentration of indoor mycoflora was obtained. The reason for this could be attributed to the aforementioned operations which were not the same for this study where no loading or unloading of grains was held at the point time of sampling. But this was experienced in November in a groundnut warehouse and could be the reason for the highest mould count.

This study agrees with the findings of Lanier *et al.* (2010) and Adhikari *et al.* (2004) where elevated levels above the permissible limits of total fungal particles in indoor agricultural settings were observed. Therefore, the working environment is unsafe and hence, the need for the reduction of mould air-mass concentration to a possible minimum and enforcement of the use of face masks and other personal protective wares while working in grain stores.

All the mould isolates from the groundnut stores were *A. parasiticus* and those from the maize and groundnut warehouse were *A. flavus*. These could be attributed to the predominating fungal contaminants of the stored grains which may also be primarily from the farmland from which the grains were grown. The predominant mould species in

particular farmland will likely be the dominating contaminant of the grown produce (Udomkun *et al.*, 2017).

The aflatoxin-producing ability of the mould isolates shows that the grain stores/warehouses constituted airborne aflatoxin. This is because aflatoxin is carried into the air by fungal spores and mycelial fragments (Mayer, 2016). Therefore, inhalation of air in the grain stores used in this study could pose a risk of aflatoxin intoxication to grain tradesmen. Moreover, the differences in the degree of fluorescence (which is directly related to the amount of produced aflatoxin) could be attributed to the sclerotial size of the strains where those with small and large sclerotia produce large and small amount of aflatoxin respectively (Benkerroum, 2020).

Aflatoxin-biosynthesis gene(s) were detected in the *Aspergillus* species isolates showing aflatoxin production ability on NRDCA. This indicates the presence of the aflatoxin gene cluster and its involvement in the aflatoxin biosynthesis observed on NRDCA (Yu *et al.*, 2004). None of the three targeted genes was amplified in isolate WG1, which did not also produce aflatoxin on culture. This could be because the isolate did not possess the aflatoxin biosynthetic gene cluster; thereby resulting in its inability to produce the toxin (Yu *et al.*, 2004). This result is in agreement with the findings of Davari *et al.* (2014), Hussain *et al.* (2015) and Dooso *et al.* (2019) who reported that they detected one or some genes involved in aflatoxin biosynthesis from aflatoxin-producing mould isolates. In this study, the *aflD* gene was amplified from all the aflatoxin-producing mould isolates, and it was the most amplified in studies by Davari *et al.* (2014), Hussain *et al.* (2015) and Dooso *et al.* (2019).

CONCLUSION

The mean mycofloral concentration in grain stores and warehouses was 2.77×10^3 – 4.05×10^3 CFU m^{-3} . This exceeded the limit of 500 CFU m^{-3} for total mycofloral concentration for agricultural and industrial environments. A total of twelve (12) isolates of *A. flavus* were obtained from the indoor air of the grain stores and eleven (11) of the twelve (12) isolates (91.67%) were confirmed to be aflatoxigenic on NRDCA. Therefore each isolate presents $30 \text{CFU}m^{-3}$ mould particles in the stores and warehouses. The aflatoxin-biosynthetic genes, *aflD* (*nor-1*), *aflM* (*ver-1*) and *aflR* were detected in *Aspergillus* species with *aflD* (*nor-1*) gene being detected in all the aflatoxin-producing species of *Aspergillus*.

Recommendations

Further studies should be conducted to quantify aflatoxin from indoor air to determine the air quality and associated risks to the community residents. Educating the farmers/workers in occupational settings about the possible presence of airborne mycotoxins will reduce the exposure risk; this can be achieved through enlightening the community about the risk associated with aflatoxin inhalation and the use

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