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Mycotoxigenic *Aspergillus flavus* from ginger and turmeric consumed in the Niger Delta Region of Nigeria

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Abstract

Ginger and turmeric sold in the open markets and retail outlets in southern Nigeria were sampled between April and August, 2017. This period coincided with the first bimodal peak of the rainy season of the 2017 cropping season. Malt extract agar (MEA) and Dichloran 18% glycerol (DG18) media were used to isolate fungi from samples with or without surface sterilisation. *Aspergillus* spp isolated were examined for the production of orange-yellow pigmentation and blue fluorescence on the reverse side of the plate on CAM under UV light. Aflatoxin production by *Aspergillus flavus* on yeast extract sucrose (YES) was verified quantitatively using High Performance Liquid Chromatography (HPLC). Data showed that *Fusarium*, *Penicillium* and *Aspergillus* spp were the dominant fungal flora. Toxigenic isolates of *A. flavus*; AFg_1, AFg_3, AFt_1, and AFt_3 produced both orange-yellow pigmentation and blue fluorescence on CAM. The production of AFB_1 and AFB_2 on YES medium was confirmed using HPLC. The occurrence of toxigenic fungi indicates that there is a potential risk of mycotoxin contamination in ginger and turmeric consumed in southern Nigeria and problems can arise from contamination with aflatoxins.

Keywords: aflatoxin, *Aspergillus*, ginger, mycotoxin, toxigenic, turmeric

Introduction

Ginger and turmeric are among the commonly used spices in the world. India is the largest producer, consumer and exporter in the world and contributes more than 50% of the world trade (Chaudhary *et al*. 2006). Other major producers are China, Nepal, Nigeria and Indonesia. These spices are obtained from underground rhizomes, and have medicinal properties (Olojede *et al*. 2009; Karim *et al*. 2010; Singletary, 2010) and also used widely for other household purposes (Sherman & Billing 1999). Farmers' interest on large scale production of these crops the world over have been stimulated by recent studies on the efficacy of these spices on the treatment of health conditions such as

diabetics, nausea, vomiting, high cholesterol, high blood pressure and inflammation (Chen *et al*. 2007; Ernest & Pittler, 2008; Kim *et al*. 2008). The Nigerian government, research institutions and many private organisations are now investing in the cultivation and marketing of these products and this has increased its trade in most Nigerian markets. In Nigeria, for instance a sizable proportion of these products are lost due to fungal contamination during the journey from the north where they are largely produced to the southern parts where they are largely consumed. Handling, processing and storage have not been the best. The way these products are displayed in Nigerian markets encourages the growth of moulds and thus mycotoxin contamination is imminent. The rain forest ecological zone of Nigeria, which is the dominant ecology in the south characterized by high temperature and humidity encourage the growth of toxigenic mould and mycotoxin production (Marin *et al*. 1998; Schmidt-Heydt *et al*. 2010).

Mycotoxins are secondary metabolites of fungi produced and secreted into a wide range of foods and food products. The most common fungal contaminants of spices belong to *Aspergillus, Penicillium* and *Fusarium* genera and some species of these genera have the ability to produce different mycotoxins such as aflatoxins (AFs) (Magan *et al.* 2011), ochratoxin (OTA) (Sultan & Magan 2010), citrinin (CTN) (Jeswal & Kumar 2014; 2015) and deoxynivalenol (DON) (Nicholson *et al.* 2007). Aflatoxins are secondary metabolites produced by the *Aspergillus* genus and they are carcinogenic (Liu & Wu, 2010). Aflatoxin contamination of food products has received serious attention because AFB_1 is classified as a Class 1A carcinogen and the EU and many countries of the world have strict maximum levels of contamination (European Commission, 2006; 2010). In 2017, WHO identified aflatoxins (Class 1A carcinogens) as responsible for causing stunting of children in lower middle income countries, especially in parts of sub-Saharan Africa. The consumption of ginger and turmeric has increased steadily, especially in Nigeria because of their health benefits and industrial uses (Bakht *et al*. 2012; 2013). Therefore, understanding the status of mycotoxin producing fungal species especially in the humid tropics where the environmental conditions are most favourable is very important. Scanty information is available on the incidence of mycotoxigenic moulds in southern Nigeria which has a high rate of consumption of these products, and this has necessitated the need for this study. The objectives of the study were to identify the fungal flora associated with commonly marketed ginger and turmeric in southern Nigeria and to ascertain the occurrence of aflatoxin producing isolates of *Aspergillus* spp from these products.

Materials and methods

Sampling

Ginger and turmeric samples were sourced from different randomly-selected retail outlets including supermarkets, grocery stores, open market stalls and wholesale outlets in Abia, Akwa Ibom, Bayelsa, Cross River, Imo and Rivers States in the Niger Delta region of Nigeria. The Niger River is located in the *Gulf of Guinea* in *Nigeria* with latitude 5° 19' 20.40" N and longitude: 6° 28' 8.99" E. Sampling was done between April – August which coincides with the first bimodal peak of the rainy season of the 2017 cropping season. A total of 400 samples were collected (240 ginger and 260 turmeric), air dried appropriately and stored in sterile cellophane bags at 4°C.

Fungal isolation and identification

Two media [Malt extract agar (MEA) and Dichloran 18% glycerol media (DG18; both Oxoid Ltd., UK)] were used to plate 2 mm ginger and turmeric pieces (5 pieces per Petri plate x 10 plates per medium) either unsterilized or after surface sterilisation (to compare the level of secondary contaminants) using 1% sodium hypochlorite for 1 min followed by rinsing in sterile water for 30 seconds. The samples were dried on filter paper. The pieces were placed equidistant on the media using a sterile forceps and incubated at 25±2°C for 10 days. Colony forming units of fungi were sub-cultured until pure cultures were isolated. The identification of the fungal flora was realized microscopically, based on the morphology of spores and conidial, colony growth and with reference to Pitt & Hocking (2009). *Aspergillus flavus* strain (NRRL 3357) was also used for comparison. Fungal colonies were

counted and recorded and separated into species. The total number of fungal colonies grown on the same set of plates was recorded. Percentage of occurrence of each fungal species was calculated.

Qualitative assay for aflatoxin production

Aflatoxin and ochratoxin screening with Coconut Agar Media (CAM) was used in the experiment (Davis *et al.* 1987). 250 mL of coconut cream (Sainsbury's, UK) was mixed with 250 mL of deionised water using a heated stirrer at 75°C. 10 g of Technical Agar No.3 (Sigma Aldrich, USA) and 0.16 g of chloramphenicol were added to the solution. The medium was autoclaved and poured into 9 cm Petri plates. After cooling at room temperature, the plates were inoculated with a spore suspension of six isolates each of *A. flavus* (AFg_1, AFg_2, AFg_3, AFt_1, AFt_2 and AFt_3 and *A. niger* (ANg_1, ANg_2, ANg_3, ANt_1, ANt_2 and ANt_3) with a sterile loop. The inoculated plates were incubated at 25°C for 7 days. They were examined for orange-yellow pigmentation and at the reverse side the presence or absence of a blue fluorescent ring under UV light, characteristic of aflatoxin and ochratoxin-producing strains (Lin & Dianese 1976). For comparison, *Aspergillus flavus* strain (NRRL 3357) and *Aspergillus carbonarius* (Carbo 197) were used as control.

Quantitative analysis using High Performance Liquid Chromatography (HPLC)

Aspergillus flavus isolates (AFg_1, AFg_2, AFg_3, AFt_1, AFt_2 and AFt_3) were subsequently cultured on a mycotoxin conducive Yeast Extract Sucrose agar medium (YES, Oxoid Ltd., UK) for 10 days. Six discs (4 mm diameter) of each fungal culture were placed in 2 mL volume safe-lock Eppendorf tubes. The weight of Eppendorf and Eppendorf + agar was taken. Aflatoxin was extracted with 0.75 mL of 100% methanol by shaking well for 1 h at 150 rpm at 25°C. The methanol extract was transferred to Eppendorf tubes and dried at 45°C in a speed vacuum. Samples were dissolved in 0.5 mL of methanol:water (50:50), vortexed and filtered with 0.22 µm filter (Kromega, Jaytee Biosciences Ltd., UK) into sylinazed HPLC vials using 1 mL syringes (Terumo Medical Corporation, UK). The HPLC equipment used was an Agilent 1200 Series system (Agilent, Berkshire, UK) with a fluorescence detector (FLD) (Millipore Waters, Corporation Massachusetts, USA), at excitation and emission wavelengths of 365 and 440 nm respectively, and flow rate of the mobile phase (methanol/water/acetonitrile, 30/60/15, v/v/v) of 1 mL/min for a running time of 12 min. Separation was achieved through the use of a C_{18} column (Poroshell 120 EC-C18 4.6 × 100 mm, 2.7 µm) preceded by a Phenomenex Gemini C_{18} 3 mm, 3 µm guard cartridge) (Castano *et al*. 2017).

Results and discussion

Occurrence of fungal flora

Aspergillus spp were isolated from ginger and turmeric samples in both media and methods at varying degrees. In direct planting method, *Aspergillus* spp accounted for between 23% (MEA) (Fig. 1a) and 43% (DG 18) (Fig. 1b) in ginger and 68% (MEA) (Fig. 2a) and 64% (DG18) (Fig. 2b) in turmeric of the total fungi species isolated. *A. flavus* and *A. niger* dominated in MEA medium in both samples accounting for more that 30% when the samples were surface sterilised. Six isolates each of *A. flavus* (AFg_1, AFg_2, AFg_3, AFt_1, AFt_2 and AFt_3) and *A. niger* (ANg_1, ANg_2, ANg_3, ANt_1, ANt_2 and ANt_3) were subsequently purified and used for further analysis. This included three each from ginger and turmeric. The AFg and AN*g* and AFt and AN*t* designate represented *A. flavus* and *A. niger* isolates from ginger and turmeric, respectively. *A. tamarii* and *A. terreus* appeared to have grown better on DG 18 medium especially in turmeric samples. Growth of *A. terreus* was not observed in ginger when the samples were surface sterilised as it seems to be a secondary infection. Other fungal species isolated from both spices in both media included; *Alternaria* sp, *Fusarium* sp, *Mucor* sp, *Penicillium* sp, *Rhizopus* sp and *Eurotium* sp (Fig. 1 & 2). In general *Fusarium* and *Penicillium* spp were the dominant fungal flora in both spices. Rawat *et al*. (2014) have reported some of these fungi in stored spices. They observed that *A. niger, A. flavus, Mucor* and *Rhizopus* species were the dominant mycoflora. Jeswal & Kumar (2014; 2015) also reported the occurrence of *F. oxysporum* and *Aspergillus* spp in both stored ginger and turmeric samples with ginger having high aflatoxin contaminations. In their study, *A. tamarii* was only confined to red chilli samples, unlike in the present study where

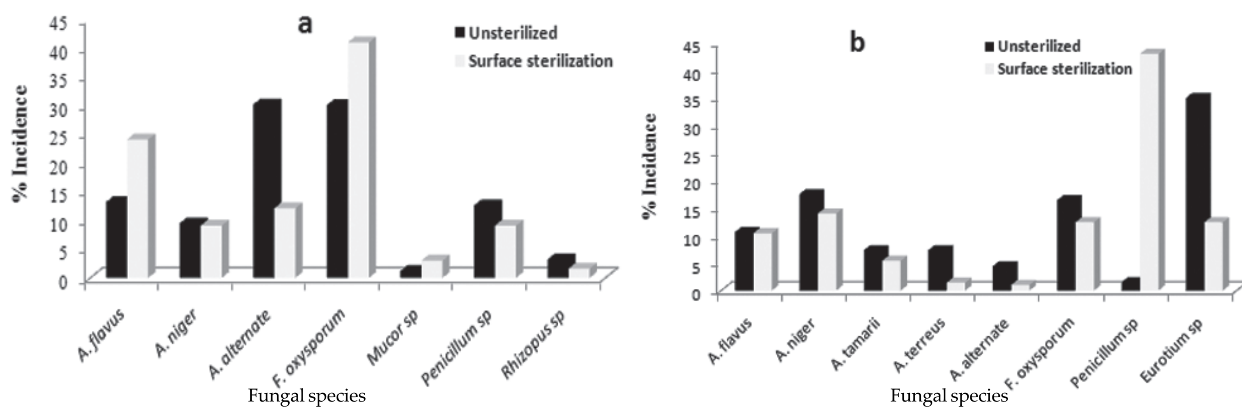

Fig. 1. Fungal species isolated from unsterilized and surface sterilized ginger samples in (a) MEA and (b) DG 18 media.

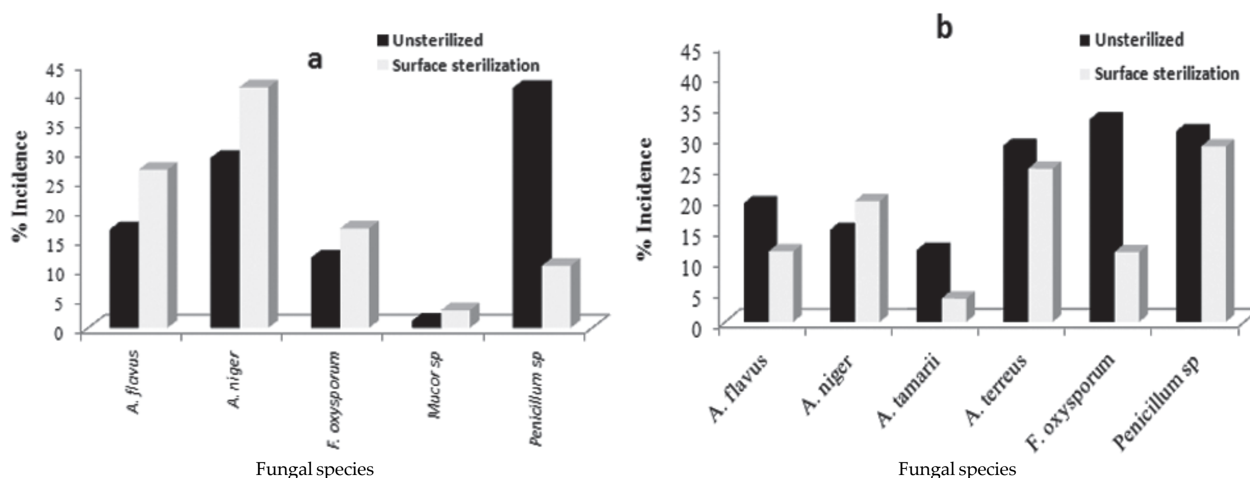

Fig. 2. Fungal species isolated from unsterilized and surface sterilized turmeric samples in (a) MEA and (b) DG 18 media.

these organisms were found in both ginger and turmeric. This may be the first report of the occurrence of *A. terreus* and *A. tamarii* in ginger and turmeric in Southern Nigeria.

Toxin production by isolates of A. flavus

Results of tests with CAM and YES media for HPLC analysis are presented in Tables 1 & 2. The *A. flavus* isolates (AFg_1, AFg_3, AFt_1 and AFt_3) produced AFs both qualitatively and quantitatively. It was necessary to carry out quantitative analyses on *A. flavus* isolates as the CAM media may not be able to identify isolates which were very low producers of AFs (Lin & Dianese 1976). None of the *A. niger* isolates produced the orange-yellow pigmentation or blue fluorescence on the reverse side of the colony under UV light, suggesting that they may be *A. niger* (uniseriate) or low toxin-producers, although this was not confirmed quantitatively. Sultan & Magan (2010) found isolates of *A. niger* from Egyptian peanuts to be non OTA producers and this is in line with the results of the present study. However, *A. niger*, *A. ochraceus* and *A. carbonarius*, have been reported to produce ochratoxin A in green coffee (Taniwaki *et al*. 1999). In the control, *A. flavus* NRRL 3357 and *A. carbonarius* Carbo 197, both rapid growers and relatively potent aflatoxin and ochratoxin producers respectively, had blue fluorescence produced on the reverse side of the colonies in 2 days. The other aflatoxin producers (AFg_1, AFg_3, AFt_1 and AFt_3) took 4–5 days to produce a blue fluorescent ring surrounding the colony. At incubation longer than 7 days, it was difficult to evaluate because mycelial growth reached the

Table 1. Production of orange-yellow pigmentation and blue fluorescence by isolates of *A. flavus* and *A. niger* on coconut agar medium (CAM).

A. Flavus isolate	Orange-yellow Pigmentation	Blue fluorescence
AFg_1	+	+
AFg_2	-	-
AFg_3	+	+
AFt_1	+	+
AFt_2	-	-
AFt_3	+	+
ANg_1	-	-
ANg_2	-	-
ANg_3	-	-
ANt_1	-	-
ANt_2	-	-
ANt_3	-	-
NRRL 3357	+	+
Carbo 197	+	+

+ = presence; - = absence; AFg = *A. flavus* isolate from ginger; AFt = *A. flavus* isolate from turmeric; ANg = *A. niger* isolate from ginger; ANt = *A. niger* isolate from turmeric.

Table 2. Production of aflatoxin B_1 and B_2 by isolates of *A. flavus* on Yeast Extract Sucrose (YES) medium

A. flavus	Toxin in ng g^{-1} of agar	
	AFB_1	AFB_2
AFg_1	192	55
AFg_2	-	-
AFg_3	551	119
AFt_1	114	22
AFt_2	-	-
AFt_3	373	547

AFg = *A. flavus* isolate from ginger; AFt = *A. flavus* isolate from turmeric; - = no toxin produced.

margin of the plate and made the blue fluorescence invisible and this was the case with the other isolates. 3-5 days was ideal for the evaluation of the plates, and a plate was assumed to be negative when on the reverse side, a thin blue fluorescent ring just external to the colony was absent.

According to Lin & Dianese (1976), the production of an orange-yellow pigmentation of the mycelium prior to the appearance of blue fluorescence is an estimator of toxin producing isolates. This was used to estimate the aflatoxin-producing ability in their study without the use of a UV lamp. The present study corroborated this, as all the isolates that produced orange-yellow pigmentation also produced blue fluorescence and was later confirmed to be toxin producers. Thus, it was found that the production of orange-yellow pigment seemed to be a reliable indicator of aflatoxin-production contrasting the works of Davis *et al*. (1987). Davis *et al*. (1987) in their work found that some isolates which produced the orange-yellow pigment did not produce blue fluorescence and concluded that the production of orange-yellow pigmentation might not be a reliable indicator of aflatoxin-producing ability. Coconut cream was satisfactory in supporting growth and blue fluorescence when used as medium. These products contained appreciable levels of added sugar; emulsifier: mono and diglycerides of fatty acids and stabilizers. *A. flavus* isolates grew extremely well on the YES medium and chemical analysis confirmed the production of aflatoxin (Table 2). The maximum accumulation of AFB_1 and AFB_2 (551 and 549 ng g^{-1}, respectively) was observed in isolate AFg_3 and AFt_3, respectively, while the least was observed in AFt_1 (114 and 22 ng g^{-1}, respectively). The most commonly found aflatoxin in food and food products is AFB_1, followed by AFB_2, AFG_1 and AFG_2 (Castano *et al*. 2017) and EU has set legislative limit for total AFs/AFB_1 in ginger, turmeric and other spices. For ginger and turmeric, the legislative limit is 5 μg kg^{-1} and 10 μg kg^{-1} for AFB_1 and total AFs, respectively. The amount of AFB_1 produced by these isolates in YES medium exceeded the EU limits and these doses raise safety concerns for ginger and turmeric and emphasize the possible hazardous nature of such contamination in the study area.

In conclusion, the current study demonstrated that ginger and turmeric consumed in the Niger Delta region of Nigeria supported the growth of toxic fungi. All the samples were contaminated with *Aspergillus* spp and most of isolated *A. flavus* were aflatoxin producing. The use of CAM media proved to be a rapid and simple method to qualify AF-producing *A. flavus* isolates. This may be a first report of *A. terreus* and *A. tamarii* occurrence in ginger and turmeric from the Niger Delta region of Nigeria. Poor post-harvest handling and storage of ginger and turmeric in the Niger Delta of Nigeria could increase the risk of contamination with aflatoxins above the legislative limits.

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