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Community Structure of Aflatoxin Producing Fungi in Cassava Products from Nigerian Geo-political Zones

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Abstract
One of the internationally recognized household food and feed safety challenge is the global contamination of agricultural commodities with aflatoxins. Fungal growth and aflatoxin contamination are the consequence of interactions among the fungus and the host crop when interplayed with favorable changes in environment/climate. Population distribution of fungi, particularly Aspergillus in substrate such as cassava products, as well as their aflatoxin contamination levels, were investigated across the six geo-political zones of Nigeria. Standard mycological methods were used to isolate and identify the fungi while Enzyme-Linked Immunosorbent Assay (ELISA) was utilized in the analysis of aflatoxins. According to results, mean fungal count ranged from $1.2x10^3$cfu/g to $1.1x10^7$cfu/g, with no significant difference ($p>0.05$) across the zones in the pellets, industrial cassava flour and Garri, except for Local Cassava flour where a highly significant difference ($p<0.01$). Species of fungi identified include; Aspergillus Sect.Flavi, Aspergillus niger, A. nidulans, A. tereus, A. fumigatus, Penicillium italicum, P. oxalicum, P. sp,. Mucor mucedo, M. sp., Neospora sp., Choanophora sp., Cladosporium sp., Rhizopus sp., Rhodotorula sp., Saccharomyces cerevisiae, Fusarium oxysporium, Botrydoplodia theobromae, Helminthosporium sp. and Trichoderma sp. A. niger had highest incidence of 9.8%, A. Sect. Flavi 8.5% and Trichoderma sp occurring least. Aflatoxin levels was highest in local cassava flour from Benue (83.54ppb±2.95). Only 10% of the cassava products met EU standard limit while 75% met US/Nigerian standard. Cassava acts as a good substrate for fungal growth and aflatoxin production, thus should be given adequate intervention attention like maize and groundnuts.

Key words: Bacteriological, Physicochemical, *Irvingia spp,* Benin

Introduction
Fungal invasion of agricultural crops is a source of concern to mankind and food safety organizations due to their specific health/economic impacts on human consumers, animals and national economy. The colonization of crops by fungi in the field, at harvest, during storage and distribution to final consumers can result in crop destructions, discolorations that lead to loss in value, diseases and mycotoxin production, the most notorious being aflatoxins. The Food and Agriculture Organization has estimated that 25% of the world's crops are contaminated by mycotoxins each year, with annual losses of around 1 billion metric tons of foods and food products. Aflatoxins are highly potent toxic fungal metabolites produced by some strains of *Aspergillus flavus*, A. *parasiticus* as well as related species like *A. nomius, A. niger, A. terreus and A. oryzae* (1,2)

Fungal growth and aflatoxin contamination are the consequence of interactions among the fungus, the host (crop or substrate) and the environment. The appropriate combinations of these factors determine the infestation and colonization of the substrate, as well as the type and amount of aflatoxin produced. The strain and distribution of the producing fungi primarily determines level of aflatoxin contamination and not necessarily the fungal count or profile. However environmental factors such as climate, storage conditions, rodent damaged of crops enormously influence both fungal proliferation and aflatoxin production. Tropical conditions such as high temperature and moisture, seasonal rains and flash floods encourage fungal proliferation and aflatoxin production (3).

Aflatoxin contamination can result in direct economic impact through export ejection from importers with stringent aflatoxin regulations such as the European Union (EU) countries. Between 2007 and 2012 alone the EU has issued 346 notifications to African countries. In Africa, it contributes to the inability of most African countries to access high-value international trade markets.

Though, maize and peanuts are the main source of human exposure to aflatoxin largely because they are the most susceptible crops to its contamination and happen to be the agricultural crops that are highly consumed worldwide (4), other crops such as cassava, that support growth of the producing fungi, under conducive environmental conditions are also being heavily contaminated with aflatoxins.

Cassava is one of the major sources of farm income and it is an important food security crop for the people of Africa. It is a staple food that provides carbohydrates or energy for over 2 billion people in the tropics. It is a higher producer of carbohydrate per hectare than the main cereal crops and can be cultivated at a consistently lower cost (5) and constitute about 40% of the food calories consumed in tropical Africa (6). Almost all the cassava produced is for

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human consumption while less than 5% is used in the industry. Also, the use of cassava flour as a raw material for the bakery and pastry industries is fast growing and gaining recognition as a reliable partial substitute for wheat cost (5).
Nigeria is currently the largest producer of cassava in the world with an annual output of over 34 million tons of tuberous root and it plays a dominant role in the rural economy in the southern agro-ecological zone (7). A lot of the researches on the occurrence and control of aflatoxins have targeted maize and peanuts, being the most susceptible, but little work has been done with other crops, especially the staples, which can pose a high exposure risk due to the frequent rate of consumption. One of such staple foods is cassava, hence this study.
This work seeks to examine the community structure and distribution of aflatoxin producing fungi in local cassava productss from climatically variable geo-polital zones of Nigeria, with a goal of identifying the zone(s) that need more control interventions.

Materials and Methods

Study Areas

Random sampling was carried out in selected states from the six (6) geo-political zones of Nigeria. Samples of local cassava products were obtained from major markets of the representative states. These were obtained from three main markets to form a sample pool and required aliquots were taken as representative test samples. The cassava products of interest were collected from Enugu State representing the South-East zone, Edo State representing the South-South zone, Ondo State representing the South-West while Gombe, Katsina and Benue States represented the North-East, North-West and North-Central zones respectively.

Experimental Design

This study was done as a six by four by three randomized setup where four kinds of indigenous cassava products were sampled from three major markets in the six geo-political zones of Nigeria, with climatic variations in the zones of study expected to influence fungal and aflatoxin contamination.

Sample Materials:

Local cassava products analyzed were cassava pellets, Local cassava flour, Industrial cassava flour and Garri.

Sample Collection

Representative samples were randomly collected during rainy and dry seasons in order to evaluate possible effect of seasonal variations in aflatoxin content in the food samples. These were obtained with polyethylene containers and taken to the laboratory for analysis. When immediate analysis was not possible, samples were preserved by reducing the moisture content to below 13% (to hinder the growth of the fungi and stop further production of aflatoxins) by drying at 60^{o}C for 4 hours and then, storing at 4^{o}C until needed for analysis.

Media Preparation

The media used in this study to isolate fungi from test samples were; Potato Dextrose Agar (PDA), Potato Dextrose Broth (PDB), Sabouraud Dextrose Agar (SDA), Yeast Extract Sucrose Agar (YES Agar) and Rose Bengal Agar (RBA). All media preparations were carried out in accordance with manufacturer's instructions (composition and preparation of media in appendix). Antibiotics (Streptomycin and Chloramphenicol – 50mg/L each) were introduced into the dissolved media after sterilization was carried out. Sterilization of media was by autoclaving for 15 minutes at 121°C and 15 pounds pressure.

Mycological Evaluations

Isolation of fungi / *Aspergillus* species from the various food samples was carried out using ten-fold serial dilution method and direct plating on SDA, PDA and RBA then incubating at 27 ± 2^{0}C for 72 hours. Colonies were counted and expressed in Colony Forming Units per gram (cfu/g).

Purification of Cultures

Distinct colonies were sub-cultured on Yeast Extract Sucrose (YES) agar and incubated at 27 ± 2^{0}C for 72hours. Then the various fungal isolates were passed through preliminary identification using their morphological and microscopic characteristics on viewing fungal mycelium stained with lactophenol cotton blue (8).

Identification of fungal isolates

Cultural and morphological characteristics like shapes, size, pigmentation, ornamentation and / or mode of attachment, asexual or sexual structures were determined with the aid of a microscope from observation of fungal mycelium stained with lactophenol cotton blue. Fungal keys and manuals were used for further preliminary identification of the isolates (9,10,11).

Determination of Incidence / Prevalence of Aspergillus Isolates.

The incidence of *Aspergillus* species in the food samples analyzed were assessed using the presence index and relative frequency criteria. These values were obtained using the formulae postulated by Foko (12):

Pif=Nsc / Tnse and Rif=Ncpc / Tnil

Where Pif=Presence index of the fungus; Nsc=Number of samples contaminated; Tnse=Total number of samples examined; Rif=Relative index of the fungus; Ncpc=Number of food materials contaminated by each fungus; Tnil=number of isolates obtained per zone.

Determination of Aflatoxin Content in the Various Food Samples

Aflatoxin content of the food samples were determined using Max Signal Total Aflatoxin Enzyme Linked Immunosorbent Assay (ELISA) test kits and these were purchased from BioOscientific Corporation, USA. The tests (food sample preparation, ELISA test protocols) were performed according to the manufacturer's instructions.

Sample Preparation for ELISA

Solid Samples

-1g of the sample was weighed out aseptically into a sterile plain container.
-5ml of 70% methanol was added to the weighed sample
-The sample-methanol mixture was thoroughly shaken to make a homogenous mixture
-The homogenous mixture was centrifuged at 4000 revolutions per minute (rpm) for 10 minutes
-2.0 ml of the supernatant (fluid) was measured aseptically into sterile pilot bottles.

Liquid Sample

-0.5ml of the sample was measured aseptically into a plain sterile container
-4.5ml of 70% methanol was added to the measured sample
-The mixture shaken thoroughly to homogenize
-2.0 ml of the mixture was transferred aseptically into a pilot bottle

ELISA test protocol

Measured 50 µL of each of the aflatoxin B1 standards was added into different wells in duplicate in the order from low concentration to high concentration. Then 50 µL of each of the samples were added into different sample wells in duplicate followed by the addition of 100 µL of antibody #1 which were then well mixed by gently rocking the plate manually for 1 minute. The plate was then incubated for 30 minutes at a temperature of 20-25ºC. The plate was washed 3 times with 250 µL of 1X wash solution. After the last wash, the plate was inverted and gently tapped dry on paper towels. Then 150 µL of 1X antibody #2 was added and the plate incubated at room temperature for 30 minutes with the microtitre plated covered. The plate was washed 3 times with 250 µL of 1X wash solution. After the last wash, the plate was inverted and tapped dry on paper towels. A 100 µL portion of TMB substrate and then incubated for 15 minutes. After incubation, 100 µL of stop buffer was added to stop the enzyme reaction. The plate was then read as soon as possible following the addition of the stop buffer on a plate reader 450 nm wavelength.

Total Aflatoxin Concentration Calculations

A standard curve was constructed by plotting the mean relative absorbance (%) obtained from each of reference standard against its concentration in ng/ml on a logarithmic curve.

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

The mean relative absorbance values for each sample were then used to determine the corresponding concentration of the tested sample in ng/g from the standard curve.

Statistical Analysis

Statistical analysis of all data generated in this research was performed using SPSS 15.0. Analysis of Variance (ANOVA) and Duncan's multiple comparison tests were used to compare the means of cfu/g of all fungal counts. Student's t-test was used to evaluate fungal counts and incidence in the zones (13).

Results and Discussion

The issue of food safety remains a universal concern because of the enormous hazards associated with consumption of contaminated foods. In this work, local cassava products (pellets or chips, local/industrial cassava flour and Garri) obtained from major markets of the climatically varied geo-political zones of Nigeria, were analyzed for their mycological profile and aflatoxin content. The selected food samples were cultivated on appropriate media using standard mycological techniques in order to isolate/characterize fungi in the samples and determine aflatoxin content using commercial ELISA methods.

Results of fungal counts of cassava products analyzed revealed in figure1, a highly diverse and varied pattern of fungal contamination. With values representing mean of fungal counts from three replicates, the total fungal count ranged from $1.2x10^3$cfu/g to $1.1x10^7$cfu/g in the cassava products across the zones. The cassava products were most contaminated in pellets at Enugu ($1.0x10^7$cfu/g), in industrial cassava flour at Edo (7.7×10^5cfu/g) and Garri at Enugu ($1.1x10^7$cfu/g), all having no significant difference ($p>0.05$) except for Local Cassava flour where a highly significant difference ($p<0.01$) was observed across the zones. The higher fungal counts observed in the southern states sampled may be attributable to the higher occurrence of rainfall in these zones compared to the northern zones (14) which may have encouraged proliferation fungi associated with stored foods while the highly significant difference in counts for Local cassava flour may be due to differences of local processing of the pellets into flour, as well as difference in environmental factors and contamination from equipment (15).

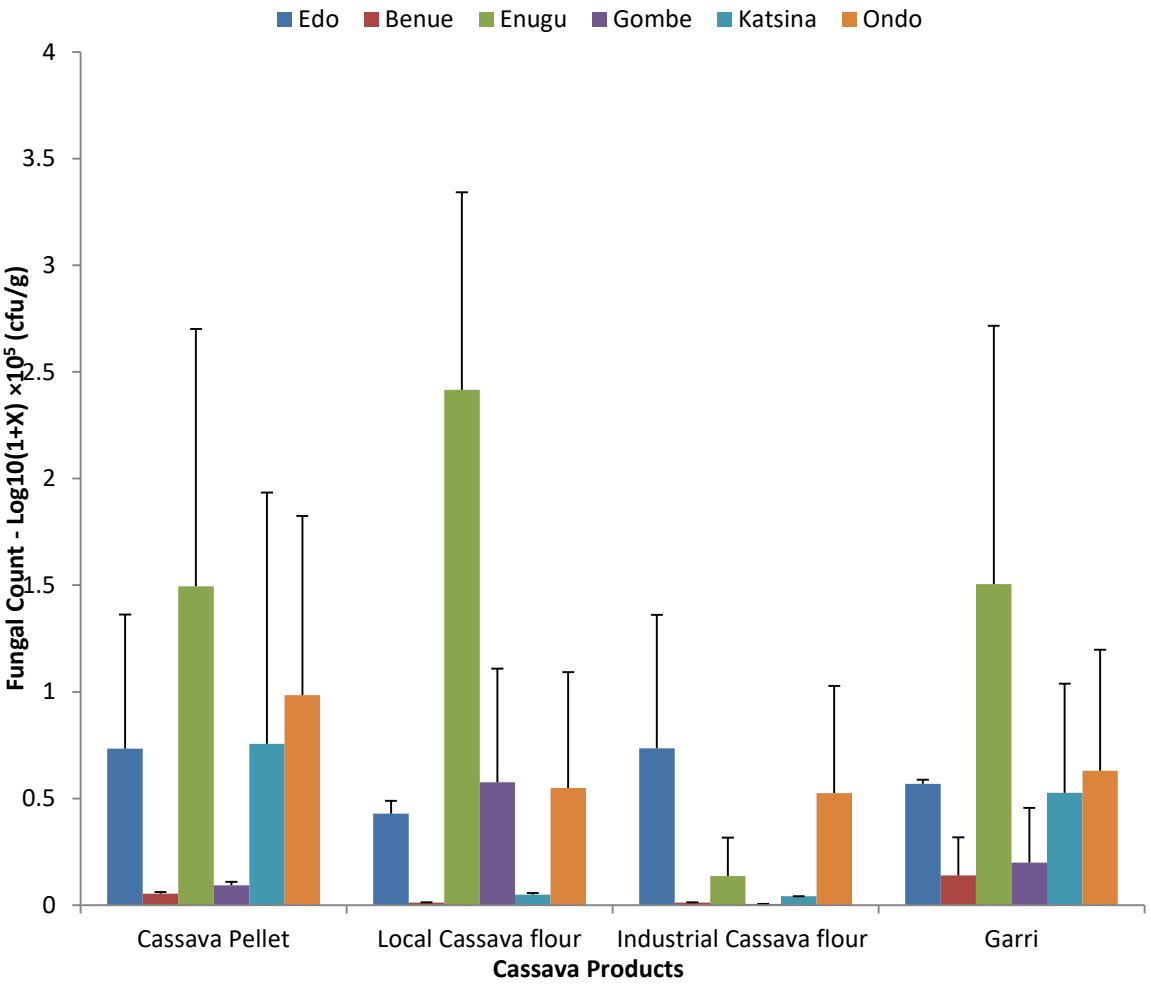

Figure 1: Mean Fungal Counts of Cassava Products from the Geo-political zones of Nigeria

Key: Zones and their representative states
North-West zone: Katsina State
North-East zone: Gombe State
North-Central zones: Benue State
South-West zone: Ondo State
South-East zone: Enugu State
South-South zone: Edo State

The fungi isolated from all the food products analyzed across the six geo-political of zones of Nigeria were identified as *Aspergillus Sect. Flavi, Aspergillus niger, Aspergillus nidulans, Aspergillus terreus, Aspergillus fumigatus, Penicillium italicum, Penicillium oxalicum, Penicillium* spp,. *Mucor mucedo, Mucor* spp., *Neospora* spp., *Choanophora* spp., *Cladosporium* spp., *Rhizopus* spp., *Rhodotorula* spp., *Saccharomyces cerevisiae, Fusarium oxysporium, Botryodiplodia theobromae, Helminthosporium* spp. and *Trichoderma* spp.
Aspergillus Sect. Flavi showed a unique pattern of distribution with a total incidence of 8.5% across the zones and having highest occurrence in Edo (SS zone), being present in all the cassava products analyzed. The incidence distribution of *A. niger* analyzed across the geopolitical zones was 9.80 % in cassava products and the organism occurred in almost all samples. *A. nidulans* had percentage occurrence as 5.6% with highest incidence from Edo samples, being isolated from all the cassava products, though was completely absent from cassava products in Katsina. *A. terreus* consistently had a low distribution across the zones but occurred more in Edo (South-South zone) than any other zone while *A. fumigatus* occurred as an uncommon contaminant across the zones though North-East zone (Gombe) was the only zone without any occurrence of this organism. The occurrence of *Aspergillus* species has recently been observed in stored grains than at harvest (16) which may lead to an increase in aflatoxin production.
The distribution of *Aspergillus* species, especially the section Flavi has been reported to significantly influence aflatoxin contamination of food products (17,18)) and these were observed to be more prevalent in the southern

zones of Nigeria. The predominant genera in Garri (fermented/roasted cassava granules) samples analyzed in this work were *Aspergillus*, *Penicillium, Mucor* and *Cladosporium* which is in agreement with the findings of Egbebi and Aboloma (19).

In this work, the occurrence of *Aspergillus flavus* in the cassava products analyzed does not however imply a corresponding occurrence of aflatoxin contamination, though circumstances that favor mold growth may also favor aflatoxin production but mold growth may occur with little or no mycotoxin production (20). The specific strains of the section Flavi group especially *A. flavus* are more important in influencing the level of aflatoxins, rather than counts or general fungal diversity. The S strains as compared to the L strains, produce high amount of toxins hence their presence may signify possible high rate of aflatoxin contamination. The predominance of L strains of *A. flavus* from *Aspergillus* sect. Flavi group in soil and maize grain samples from Nigeria has been reported in the work of Atehnkeng *et al*. (17,18). They observed a predominance of >90% L strains and <3% as frequency of occurrence of an unnamed taxon (SBG).

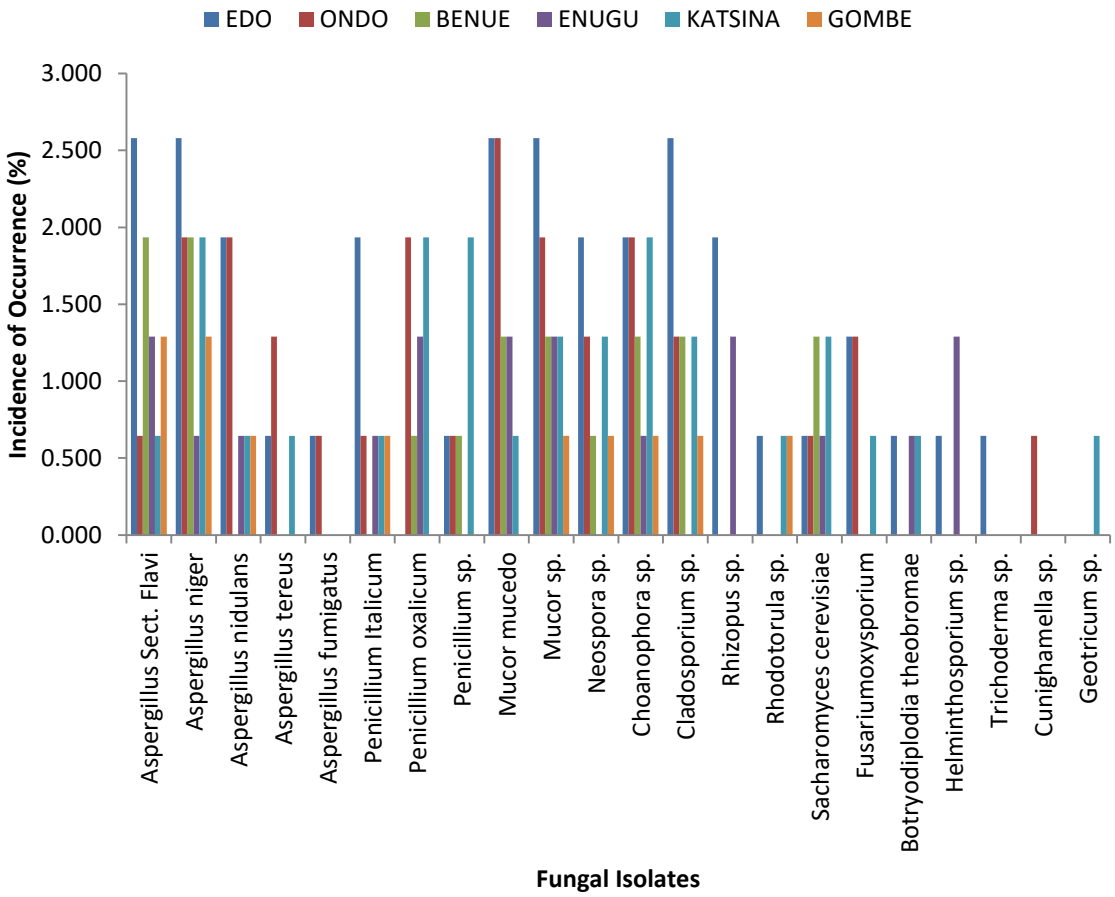

Figure2: Incidence of Fungal Isolates from Cassava Products in the Geo-political Zones of Nigeria

Key: Zones and their representative states
North-West zone: Katsina State
North-East zone: Gombe State
North-Central zones: Benue State
South-West zone: Ondo State
South-East zone: Enugu State

Aflatoxins are internationally recognized as highly toxic carcinogens that contaminate crops worldwide (21). In this research, the same cassava products (pellets, local and industrial flour, Garri) examined for fungal profile above, were also assessed for aflatoxin contamination with values illustrated in figure 3. Local cassava flour was observed to be more susceptible to aflatoxin contamination than other cassava products, the most contaminated being local flour from Benue (83.54±2.95 ppb), probably due to the crude processing and lack of Good Manufacturing Practices (GMP) during the conversion of pellets to local flour. The least contaminated cassava

product was Garri from Edo, Benue and Gombe, with no detectable levels of aflatoxins. This may be due to fermentation and a generally low moisture content attained after roasting of the cassava granules which is known to reduce fungal contamination and aflatoxin production (19). Ogiehor *et al.* (22) reported similar low levels of aflatoxin in Garri sampled from selected states in Southern Nigeria. Essono *et al*. (15) analyzed cassava chips for aflatoxin levels in Cameroun. They reported a range of 5.2 – 14.5 ppb compared to a range of 1.65±0.06 ppb – 43.11±1.52 ppb observed in this work. Highly significant difference ($p<0.01$) in aflatoxin contamination was recorded in the various cassava products across the zones which may have been due to climatic, processing and storage variations (23) in the zones.

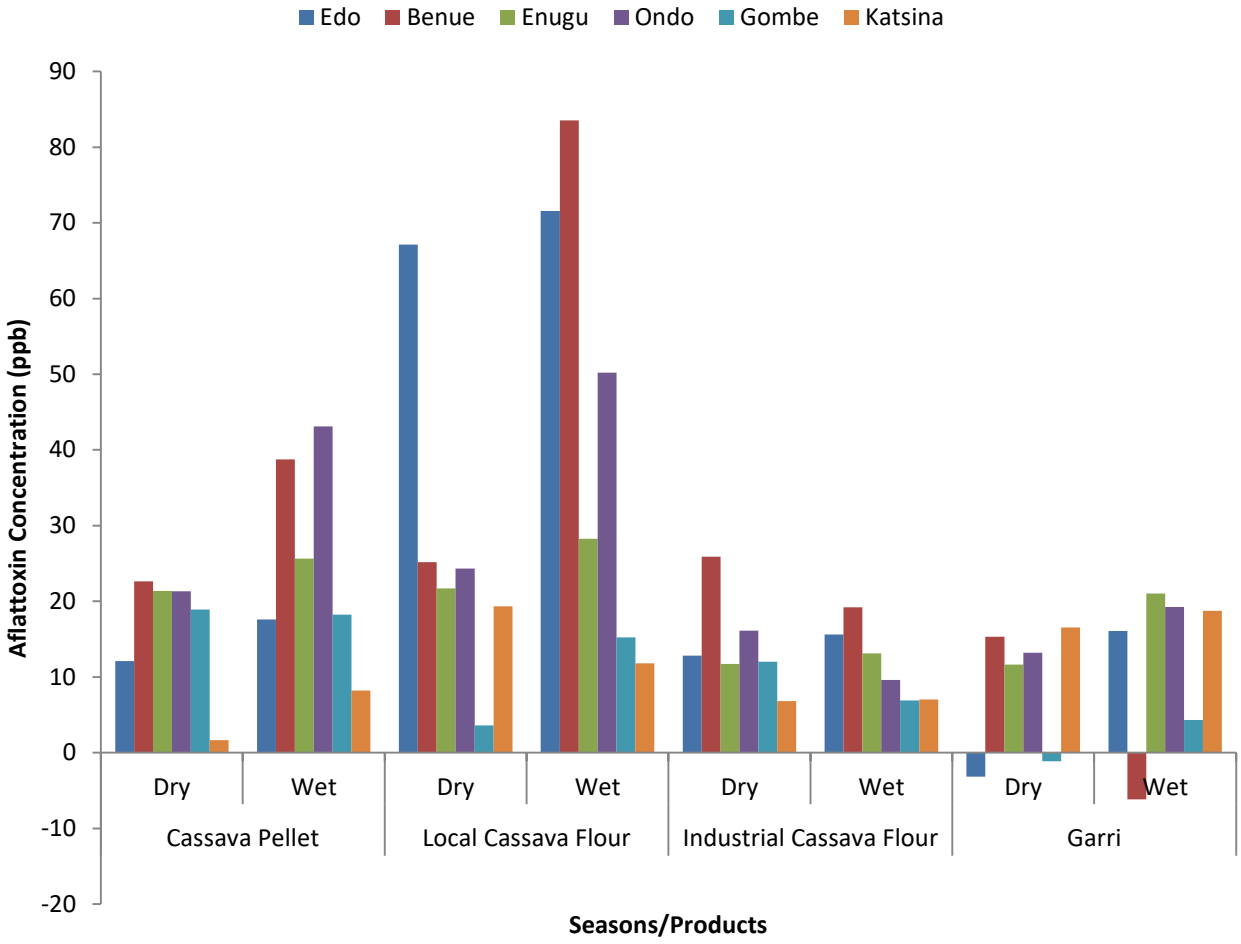

Figure 3: Total Aflatoxin Content (ppb) of Cassava Products from the Geo-political Zones of Nigeria
Key: Zones and their representative states
North-West zone: Katsina State
North-East zone: Gombe State
North-Central zones: Benue State
South-West zone: Ondo State
South-East zone: Enugu State
South-South zone: Edo State

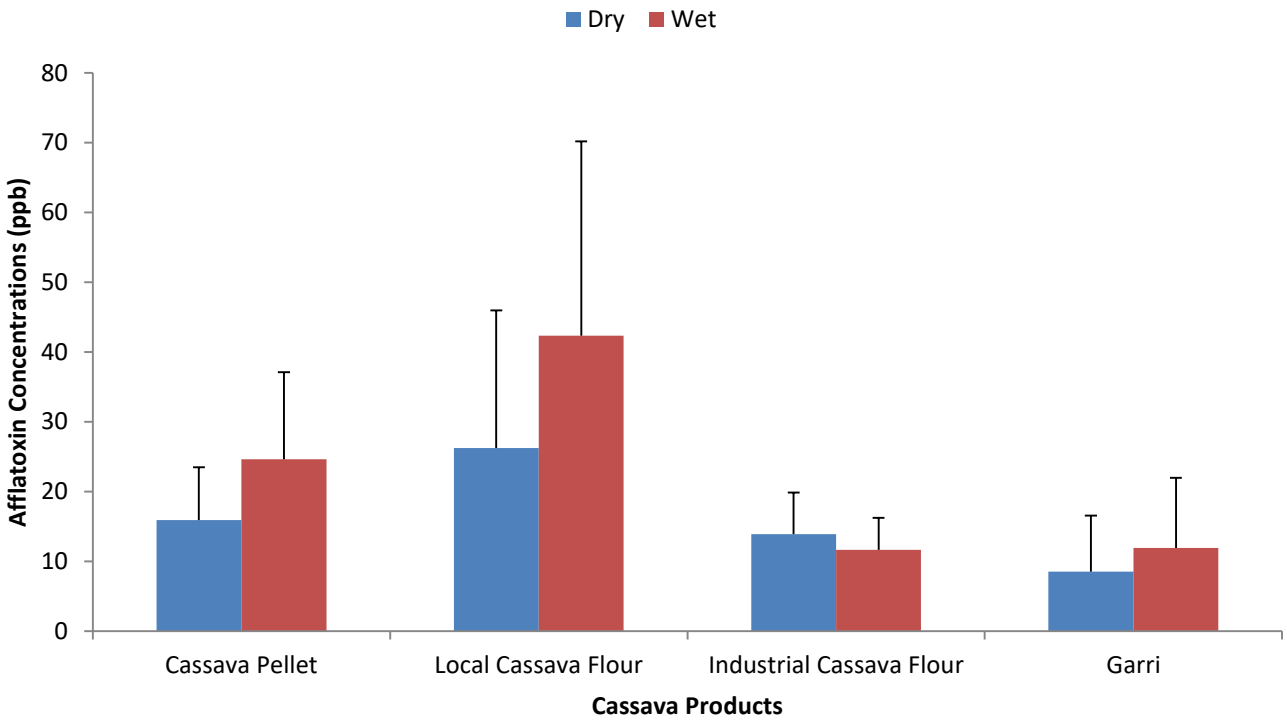

Figure 4: Effect of Season on Aflatoxin Content of Cassava Products in the Geo-political Zones of Nigeria
P>0.05 : No significant difference in Aflatoxin content

Rainfall is an important climatic factor that has been reported to influence fungal proliferation and increase aflatoxin content of food products (24**).** The mean annual rainfall in Nigeria geo-political zones showed an ordering from highest to the least to be South South, South East, South West, North Central, North East and North West with about 39% of total rainfall in Nigeria accounted for by South South zone of the country (14).

In this work, samples of cassava products were obtained during the wet and dry seasons in other to test the effect of season (Rainy Vs Dry) on aflatoxin contamination (figure 4). Aflatoxin content of most foods analyzed were higher during the rainy seasons than the dry seasons but their increase were not statistically significant ($p<0.05$). This further supports previous observations in this research that higher fungal counts and aflatoxin contamination may be observed in the southern zones due to higher amount of rainfall (14) probably as a result of increase in moisture content of the products, especially during storage (25). In Nigeria, the southern zones are known to store food products much longer than the northern zones which are the agricultural factories of the nation.

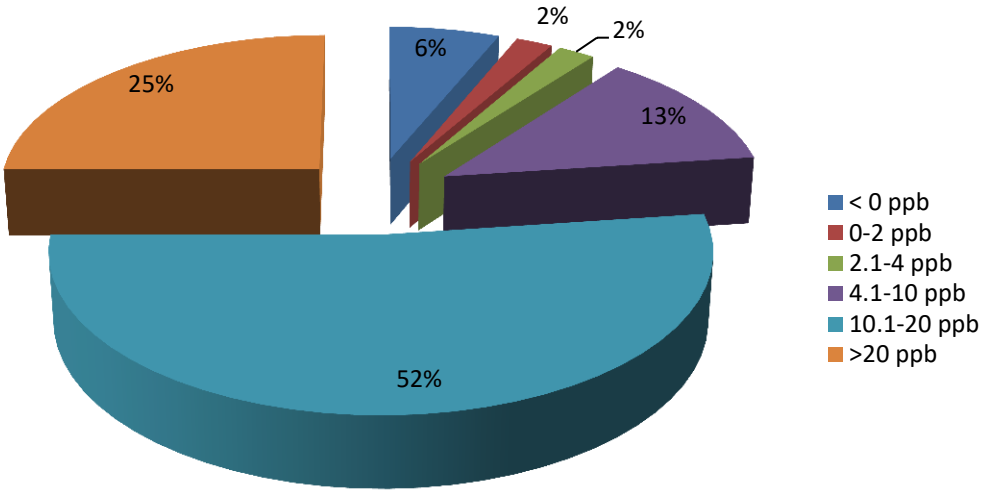

Figure 5: Aflatoxin Contamination of Cassava Products in Relation to National and International Standards

Several efforts by national government of nations, non-governmental organizations and individuals, to eliminate its occurrence in food products have proved inadequate. Hence, governments of various countries have established or proposed regulations for controlling aflatoxin levels in foods and feeds (26). Nations worldwide have stipulated maximum allowable limits of aflatoxins in foods as a way to reduce the hazards associated with its consumption. The European Union (EU) has standard limit of 2ppb of AFB1 and 4ppb for total aflatoxins human foods. United States of America have a limit of 20ppb which is similar to that of Nigeria.

Data from this work shows that in Nigeria, only a tragic 8% of cassava products examined met EU standard though about 75% met the Nigerian/US standard of 20ppb. This has high economic consequences. Because of the frequency of consumption of cassava products in Nigeria, even the 25% having higher limit than US/Nigerian standard, is expected to have serious health hazards due to consistent moderate exposures of consumers to the deadly toxin.

Conclusion

This work revealed higher fungal counts, occurrence of possibly toxigenic *Aspergillus* species and aflatoxin contamination of cassava products in the southern zones compared to the northern zones of Nigeria. Considering that aflatoxin negatively impact on agriculture and other associated industries as well as health of consumers, special interventions targeting the peculiar nature of the southern zones of Nigeria, is needed to support ongoing control measures in the country in order to effectively reduce and ultimately eliminate aflatoxin occurrence on indigenous food and feed.

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