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Microbial Evaluation and Identification of Aflatoxin-Producing Fungi in Dried Stored Maize Samples Sold in Open Markets in Benin City, Nigeria

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Abstract: Maize (*Zea mays*) is an annual monoecious crop and the most important cereal in the world after wheat and rice with regard to cultivation areas and total production. Despite the various improvements in the drying and storage of maize, the growth of moulds is usually a common occurrence in stored grains. The grain is vulnerable to biodeterioration by mycotoxigenic fungi which include *Aspergillus*, *Fusarium* and *Penicillium*. In this study, the microbial load of dried stored maize samples from three different open markets in Benin City was evaluated, and the aflatoxin-producing *Aspergillus* specie identified. The pH and moisture contents of samples were also determined. Five fungal species; *Aspergillus niger*, *Rhizopus* sp. *Fusarium* sp. *Aspergillus flavus*, and *Penicillium sp*., as well as three bacterial species; *Staphylococcus aureus*, *Bacillus* sp. and *Pseudomonas* sp. were isolated and identified using standard cultural and morphological techniques. The mean total bacterial count ranged from $3.8\pm0.20\times10^3$ (Oba market) to $5.2\pm0.20\times10^3$ (New Benin market), while the mean total fungal count ranged from $3.7\pm0.29\times10^4$ (Oba market) to $6.47\pm0.38\times10^4$ (New Benin market). The mean pH ranged from 5.9 (Oba market) to 6.7 (New Benin market) while the mean moisture content ranged from 16.1% (Oba market) to 18.2% (New Benin market). *Aspergillus flavus* was identified as the potential aflatoxin-producing specie, and it had 100% occurrence in all samples. The study showed that stored maize samples had higher fungal load than bacteria, and were contaminated with *Aspergillus flavus*; a potential aflatoxin-producing fungus. It is then necessary to observe strict pre and postharvest practices in order to reduce microbial contamination of stored grains of the unsuitability of the culture medium in the preliminary recovery of this unique group of herbicide degrading bacteria.

Keywords: *Aspergillus flavus*, Aflatoxin, Maize, Storage, Evaluation

Introduction

Maize (*Zea mays*) is an annual monoecious crop, and a member of the Poaceae family. It is the most important cereal in the world after wheat and rice with regard to cultivation areas and total production (Purseglove, 1992; Osagie and Eka, 1998). It serves as a staple food for more than 300 million of Africa's most vulnerable inhabitants, and the most important staple food on the continent (Ogunbodede and Olakojo, 2001). It also serves as a raw material on which many agro-based industries depend. The Food and Agricultural Organization (FAO) 1996 reported that a total of 5.5 million tons of maize was produced in Nigeria in 2005. For effective storage, the grains are usually dried to moisture content of 13% or below (Christensen and Kaufmann, 1974).

Despite the various improvements in the drying and storage of maize, the growth of moulds is usually a common occurrence in stored grains (Janardhana *et al*., 1999; Cardwell *et al*., 2000).

The basic problems generally associated with the storage of maize grains are moisture, attack by insects, rodents and saprophytic fungi (Atehnkeng *et al*., 2008). The grain is vulnerable to biodeterioration by mycotoxigenic fungi which include species of *Aspergillus*, *Fusarium* and *Penicillium*. Mycotoxins are toxic secondary metabolites produced by certain fungi in agricultural products that are susceptible to mould infestations (Wagacha and Muthomi, 2008; Morenoa *et al*., 2009). Mycotoxin attracts worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity and trade (Wagacha and Muthomi, 2008).

The FAO estimates that between 25% and 50% of agricultural crops worldwide is contaminated by mycotoxins (Fandohan *et al.,* 2003; Lewis *et al*., 2005; Wagacha and Muthomi, 2008). The estimated value of maize loss to

aflatoxin is $225 million per year, out of the $932 million due all the mycotoxins in the United States (Betran and Isakeit, 2003). The most important mycotoxins are aflatoxins, ochratoxins, deoxynivalenol (DON or vomitoxin), zearalenone, fumonisin. Aflatoxins B_1, B_2, G_1, and G_2 are produced by some strains of *Aspergillus flavus, A. parasiticus* and *A. nomius* with aflatoxin B_1 being the most common. Poor harvesting practices, improper storage and less than optimal conditions during transport and marketing of maize can also contribute to fungal growth and proliferation of mycotoxins (Bhat and Vasanthi, 2003; Wagacha and Muthomi, 2008). Destruction of aflatoxins by conventional food processing is difficult because they are typically resistant to heat and detection is complicated due to limitations in analytical capacity.
This study is aimed at evaluating the fungal load of dried stored maize samples from different markets, to isolate and identify aflatoxin-producing *Aspergillus* species from dried stored maize samples.

Materials and methods

Collection and purchase of maize samples: The maize samples were purchased from Uselu, New Benin, and Oba markets in Benin City. The samples were taken to the laboratory in sterile polyethylene bags.
Microbiological analyses:
Enumeration of bacteria and fungi: The total aerobic microbial count was carried out on Nutrient Agar. The differential bacterial enumeration of samples was carried out on nutrient agar with antifungal agent, Griseofulvin (manufactured by Glaxo Nigeria Ltd, Lagos.) incorporated at a concentration of 300mg to 250ml, While the selective fungal count was on Potato Dextrose Agar incorporated with antibacterial agent; streptomycin.
Serial dilution: Maize grains were ground using laboratory sterilized mortar and pestle. From each ground sample, 20 g was aseptically weighed and 180 ml of sterile distilled water was added to prepare a sample homogenate of 10^{-1} dilution. Subsequent dilutions were carried out using sterile distilled water as diluents. With separate sterile pipettes, decimal dilutions of 10^{-2} and 10^{-3} were prepared by aseptic transfer of 1.0 ml from previous dilution to 9.0 ml of the next higher dilution. Appropriate dilutions were used for microbiological analyses on Nutrient Agar and Potato Dextrose Agar to select for bacterial and fungal colonies respectively.
Microbial enumeration by pour plate method: Enumeration of bacteria and fungi was by pour plate method. One (1) ml of appropriate dilutions of maize sample was aseptically transferred into sterile well-labelled Petri dishes. Approximately 15ml portion of tempered Nutrient, and Potato Dextrose Agar were poured into the different plates, swirled to distribute the inoculum evenly, left to solidify on a level laboratory bench and incubated at room temperature (28±2°C) for 24-72 hours. The resultant fungi and bacteria colonies were counted and recorded in colony forming unit (CFU)/g.
Pure culture isolation: Streak plate method was used for pure culture isolation of bacteria and fungi. This involved streaking the initial inoculum over an area using a sterilized wire loop. The loop was then flamed severally and streaks were made through the inoculum area to the side of the plate. The needle of the loop was flamed and streaks were repeated to another side of the plate. Pure culture isolation of moulds was by picking the spores of young mycelium using sterile wire loop needle. Pure cultures of the bacteria and fungi were later stored on nutrient agar and PDA slants at 4±1°C.
Characterization and identification of microbial isolates: Both bacterial and fungal isolates were characterized prior to identification.
Characterization and identification of bacteria: The bacterial isolates were characterized using cultural characteristics, morphology of cells and biochemical tests. The cultural characteristics checked for were shape, size, colour, surface and elevation of the bacterial colonies on the medium. The morphological characteristics were examined under the microscope after staining for Gram and spore reactions. These include shape of cell (spherical or rod), arrangement of cells; either singly, in pairs, groups or in chains. Biochemical tests were carried out in order to completely identify the bacterial isolates. These tests include Oxidase, Indole, Urease, Catalase, Methyl Red, Voges-Proskauer and Citrate Utilization. The bacterial isolates were identified with reference to the 8th edition of Bergey's Manual of Determinative Bacteriology.
Characterization and identification of fungi: Fungal colonies were examined macroscopically on the PDA plates. Cultural characteristics such as shape, size, colour, surface and elevation of colonies on the medium were observed and recorded. Microscopic examination was made after staining with lactophenol blue, and morphological features such as type of hyphae, fruiting bodies and spore shape were noted. Fungi were identified based on cultural and morphological characteristics.
Physico-chemical characteristics:
pH determination: The pH value of sample homogenate was determined using a single electrode pH meter. The sample was prepared by homogenizing 20.0g of ground maize sample in 180 ml of sterile distilled water.

Determination of moisture content: The percentage moisture content of the samples was determined using the dry-oven method, following the principle of drying to constant weight. Ten (10) g of each sample was weighed, and then weight of empty crucible (W_I) was taken using a weighing balance. The samples were dried in the oven at 103°C over night. After drying, final weight (W_F) and percentage moisture content were calculated thus:

$$MC = \frac{[W_I - W_F] \times 100}{W_I}$$

where MC = moisture content, W_I = Initial weight, W_F = Final weight

Results

Microbial analysis of maize samples purchased from 3 different markets in Benin City gave fungal counts which ranged from $3.7\pm0.29\times10^4$ (Oba market) to $6.47\pm0.38\times10^4$ (New Benin market) and mean bacterial counts ranging from $3.8\pm0.20\times10^3$ (Oba market) to $5.2\pm0.20\times10^3$ (New Benin market) as shown in Table 1. Samples from Oba market had the lowest bacterial and fungal count while samples from New Benin market had the highest bacterial and fungal counts. Three bacterial species as well as five fungal species were isolated and identified.
The bacterial isolates were *Pseudomonas* sp., *Staphylococcus aureus* and *Bacillus* sp. as shown in Table 2, and the fungal isolates were *Aspergillus niger, Rhizopus* sp., *Fusarium* sp., *Penicillium* sp. and *Aspergillus flavus* shown in Table 3. Table 5 shows the occurrence of the fungal isolates and their frequency of occurrence. *Aspergillus flavus* was the most prevalent fungus on the sampled maize grains with isolation frequency of 100 %.

Moisture content and pH of dried stored maize samples: The pH values and moisture content of all the samples were analyzed and are shown in Table 4. The mean pH values of the maize samples ranged from 5.9 (Oba market) to 6.7 (New Benin market). The moisture content of the dried maize samples analyzed ranged from 16.10 (Oba market) to 18.20% (New Benin market).

Table1: Microbial counts of dried stored maize samples obtained from different open markets in Benin City

Location	**Samples**	**Bacterial count (cfu/g)**	**Mean bacterial count (cfu/g)**	**Fungal count (cfu/g)**	**Mean fungal count (cfu/g)**
	U_1	$3.90 \pm 0.33\times10^3$		$4.20 \pm 0.54 \times10^4$	
Uselu market	U_2	$3.80 \pm 0.28 \times10^3$	$4.10 \pm 0.21 \times10^4$	$4.10 \pm 0.42 \times10^4$	$4.53 \pm 0.41\times10^4$
	U_3	$4.60 \pm 0.25 \times10^3$		$5.30 \pm 0.29 \times10^4$	
	N_1	$5.10 \pm 0.21 \times10^3$		$6.30 \pm 0.37 \times10^4$	
New Benin market	N_2	$4.40 \pm 0.20 \times10^3$	$5.20 \pm 0.20 \times10^3$	$5.50 \pm 0.41 \times10^4$	$6.47 \pm 0.38\times10^4$
	N_3	$6.10 \pm 0.18 \times10^3$		$7.60 \pm 0.36 \times10^4$	
	O_1	$3.30 \pm 0.16 \times10^3$		$3.10 \pm 0.22 \times10^4$	
Oba market	O_2	$3.60 \pm 0.21 \times10^3$	$3.80 \pm 0.20\times10^3$	$3.80 \pm 0.25 \times10^4$	$3.73 \pm 0.29\times10^4$
	O_3	$4.50 \pm 0.24 \times10^3$		$4.30 \pm 0.41 \times10^4$	

Key: U = Uselu N = New Benin O = Oba market

Table 2: Cultural, morphological and biochemical characteristics of the bacterial isolates obtained from maize samples from open markets in Benin City

Characteristics	**Isolate 1**	**Isolate 2**	**Isolate3**
Cultural			
Elevation	Convex	Convex	Low convex
Margin	Entire	Smooth	Entire
Colour	Yellow	Creamy white	Light green
Shape	Circular	Circular	Circular
Morphological			
Gram staining	+	+	-
Cell type	Cocci	Rod	Rod
Cell arrangement	Cluster	Single	Single
Biochemical			
Catalase	+	+	+
Indole	-	-	-
Urease	+	-	-
Oxidase	-	-	+
Citrate	+	-	+
Glucose	+	+	+
Coagulase	+	-	-

Key: 1.*Staphylococcus aureus*, 2. *Bacillus* sp., 3. *Pseudomonas* sp

Table 3: Cultural, morphological characteristics of the fungal isolates obtained from dried maize samples from open markets in Benin City

Characteristics	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 4**
Cultural	White cotton colonies on PDA	Cotton white colonies on PDA	Greenish yellow colonies on PDA	Greenish colonies on PDA
Morphological				
Nature of hyphae	Non-septate	Septate	Septate	Septate
Colour of spore	Whitish	Whitish	Brownish	Greenish
Type of spore	Sporangiophore	Conidiospore	Conidiospore	Conidiospore
Appearance of special structures	Rhizoids	Micro and macro-conidia	Foot cells	Foot cells

Key: 1. *Rhizopus* sp., 2. *Fusarium* sp., 3. *Aspergillus flavus* 4. *Penicillum* sp.

Table 4: Mean moisture content and pH of the dried maize samples obtained from different open markets in Benin City

Location	**Samples**	**pH**	**Mean pH**	**Moisture (%) moisture**	**Mean content content**
	U_1	6.7		16.90	
Uselu	U_2	6.7	6.6	17.00	17.1
	U_3	6.3		17.50	
	N_1	6.8		17.90	
New Benin	N_2	6.9	6.7	18.10	18.2
	N_3	6.5		18.60	
	O_1	5.9		15.70	
Oba market	O_2	6.1	5.9	15.40	16.1
	O_3	5.8		17.20	

Key: U = Uselu market N = New Benin market O = Oba market

Table 5: Synopsis and prevalence rate of fungal isolates from maize samples obtained from open markets in Benin City

Fungal isolate	Market	Frequency of occurrence (%)
Aspergillus niger	New Benin , Oba	100.00
Fusarium sp.	Uselu, New Benin, Oba	55.60
Aspergillus flavus	Uselu, New Benin, Oba	100.00
Rhizopus sp.	New Benin , Oba	33.33
Penicillium sp.	New Benin , Oba	22.22

Discussion and conclusion

Maize is a staple food in Nigeria, inappropriate post-harvest practices with favourable seasonal temperature and relative humidity have accelerated the infection of grains by fungi (Dawlatana *et al*., 2002). The mean total bacterial counts in the samples ranged from $3.8\pm0.20\times10^3$ (Oba market) to $5.2\pm0.20\times10^3$ (New Benin market) while the mean total fungal counts ranged from $3.7\pm0.29\times10^4$ (Oba market) to $6.47\pm0.38\times10^4$ (New Benin market). The bacteria load in the maize samples were comparably lower than the fungal loads in the dried maize samples, this may be attributed to the low moisture content in dried sample which does not considerably favour bacterial growth. Dried stored maize samples from New Benin market had the highest bacterial and fungal counts compared to the other two markets, this could indicate that the environmental conditions and handling processes by different sellers in each of the markets may greatly affect the microbial load. Nutritional status of the dried stored maize samples may also account for the high difference in microbial load of different samples (Sullivan, 2004).

The moisture content of the samples ranged from 16.10-18.20% and was higher than the recommended 13% for safe storage (Christensen *et al*., 1974), and thus could still favour some bacterial, and fungal growth. Janardhana *et al*. (1999) also reported moisture content higher than 13% level recommended for effective storage of maize grains. This high moisture content is probably due to the combined effects of the prevailing climatic conditions (Adebanjo and Popola, 2003) and the practice of non-scientific method of handling grains during harvesting, shelling and drying. High moisture content may also be due to the storage of maize grains in synthetic bags which trap moisture and hence, an increase in the moisture content of maize grains. Mean pH values of the samples ranged from 5.9- 6.7. Bacterial and fungal load of samples increased as pH value increased. This indicates that the fungi and bacteria thrive better at pH close to neutrality in foods.

Bacteria isolates from the samples include *Bacillus* sp., *Staphylococcus aureus, and Pseudomonas* sp. They have been reported to have beneficial roles in plant life as endophytes; bacteria that enter plant parts and establish lifelong relationship with the host without harm (Ajcann, 2007). The presence of Bacillus sp. may be due to its resistant spores which aids its survival in unfavorable conditions. The presence of *Staphylococcus aureus* and *Pseudomonas* sp. in the dried stored maize samples could be attributed to contamination from human handling, the surrounding air and environment during drying and display for sale in the market. Presence of *Bacillus* sp. and *Pseudomonas* sp. in the samples taken were corroborated by the work of Figueiredo *et al*. (2009) and Rai *et al*. (2007). Fungal isolates from maize samples include *Aspergillus flavus, Rhizopus* sp., *Fusarium* sp. and *Penicillium* sp. All the fungal isolates have been previously reported (Adebanjo *et al*., 1994; Bankole *et al*., 2003; Bankole *et al*., 2004).

This study showed that dried maize grains were contaminated by microorganisms that could produce mycotoxins. Similar observations have been made in some other African countries especially on maize products (Marasas *et al*., 2001). Since most fungal spores are found in the air, the spores may contaminate the maize grains during drying. *Aspergillus flavus* was isolated from all the samples. Its presence indicates that the dried maize samples may be contaminated with aflatoxin. *Aspergillus flavus* growth and subsequent aflatoxin contamination are a consequence of an interaction between the fungi, the host (maize grain) and the environment. The appropriate combination of these factors determines the degree of the colonization of the substrate, and the type and amount of aflatoxin produced. Therefore, increase in the growth of *Aspergillus flavus* in food will trigger aflatoxin production.

It is then necessary to observe strict pre- and post-harvest practices in order to reduce fungal contamination by aflatoxin-producing *Aspergillus* species. Contamination of maize is an important but unrecognized risk to public health and can have long-term health implications (Coulter *et al*., 1986; Bucci *et al*., 1990; Rheeder *et al*., 1992; Abdulrazzaq *et al*., 2004).

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