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# Occurrence of Aflatoxins and Molecular Characterization of *Aspergillus* Section *Flavi* Isolated from Popcorn and Corn Kernels Sold in Cotonou (Benin)

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## Abstract

Popcorn is one of the most widely consumed snacks, produced by processing *Zea mays* var. *everta*. Like regular corn, this variety is susceptible to aflatoxin contamination. These mycotoxins are recognized as heat-stable compounds and could end up in finished popcorn, posing a major public health problem given the snack's popularity in Cotonou. This study aimed to examine the contamination of popcorn and corn by aflatoxins and to evaluate the aflatoxin-producing potential of *Aspergillus* section *Flavi* strains isolated from these commodities. The occurrence of aflatoxins and fungi was evaluated in 26 popcorn and 40 corn samples collected across street vending sites and markets in Cotonou, Benin. Fungal contamination was evaluated by seeding potato dextrose agar media and identifying isolates morphologically. The strains of *Aspergillus* section *Flavi* were characterized by the Polymerase Chain Reaction (PCR) of five genes within the aflatoxin biosynthesis gene cluster to assess biosynthetic potential, and Restriction Fragment Length Polymorphism (RFLP) of the Beta-tubulin gene. Thin-layer chromatography (TLC) was used to screen and identify aflatoxins from culture extracts. The most common fungi contaminating corn kernels were *Aspergillus* section *Flavi*, followed by section *Nigri*, Mucorales, and *Penicillium* spp. For popcorn, *Aspergillus* section *Nigri* was predominant, followed by section *Flavi*, *Penicillium* spp., and *Mucorales*. Thirty-one strains of *Aspergillus* section *Flavi* were isolated from popcorn and 14 from corn kernels. Molecular characterization indicated that 39 of the isolated strains belonged to species close to the *Aspergillus parasiticus* clade. Aflatoxins were detected in 61.5% (16/26) of the popcorn samples and 37.5%

(15/40) of the corn samples. It is recommended to set up a rigorous quality control system for street-vended food products to monitor and mitigate aflatoxin contamination.

## Keywords

Children, RFLP, Street Food, Corn, Mycotoxins

## 1. Introduction

Mycotoxins are secondary metabolites produced by certain mold species [1]. Most of them are toxic to humans and animals, causing various ailments sometimes leading to death [2] [3]. Among them, the most dangerous are the aflatoxins [4]. This group of mycotoxins is hepatotoxic, a primary cause of liver cancer, and can target other noble organs such as the kidneys, with severity compounded when combined with other pathologies such as HIV or hepatitis B [5]-[7]. Its presence is highly prevalent in tropical countries where hot and humid climatic conditions and poor post-harvest storage practices favor fungal proliferation and toxin production in staple food [8].

Exposure to aflatoxins has consistently been elevated among populations in sub-Saharan Africa, primarily attributable to the consumption of staple foods such as maize and groundnuts that are frequently contaminated at high level [9] [10]. Consequently, many children in this region are chronically exposed to substantial dietary aflatoxin loads, a situation that has been epidemiologically associated with impaired growth and developmental delays, as demonstrated in studies conducted across West Africa [11] [12].

This work focuses on popcorn, a snack increasingly served for breakfast as mothers face pressure to reduce time spent in the kitchen to dedicate themselves to their job or any other task that can increase the purchasing power of their household.

These popcorn products are produced and distributed within the informal sector, which lacks regulatory oversight regarding the hygienic conditions of foods sold at street vending sites. The informal food sector predominantly comprises street food vendors, which serve as a critical response to the diverse dietary needs of urban populations by providing ready-to-eat, widely consumed foods at affordable prices. However, the safety and quality of these food products remain uncertain because internal standards and regulatory frameworks to ensure consumer protection have yet to be established.

Studies conducted worldwide investigating corn-based foods have demonstrated significant mycotoxic risks. For instance, the work of [13] in Spain showed the contamination of popcorn by aflatoxins. The objective of this work is to thoroughly assess the mycological profile and aflatoxin contamination levels of finished popcorn and raw corn kernels produced and marketed in cotonou, Benin providing empirical evidence to support food safety policy interventions.

## 2. Materials and Methods

### 2.1. Sampling

The study was conducted in the urban district of Cotonou. A total of 26 finished popcorn samples were collected from street vendors and some general food shops distributed across the city. The collected items included various commercial categories: sweetened popcorn (n = 2), milk-sweetened popcorn (n = 19), artificially colored milk-sweetened popcorn (n = 4), and salted popcorn (n = 1). In parallel, 40 raw corn kernel samples were collected from some major markets. To ensure geographic representation, the study area was divided into 3 zones (Figure 1) based on neighborhood clusters structured as follows:

- Zone 1 (East Sector): comprising the neighborhoods of Vedoko, Kouhounou, Menontin, Zogbo, Fifadji and Sainte Rita for a total of 15 corn samples and 11 popcorn samples in this zone.
- Zone 2 (Central sector): comprising Etoile Rouge, Agontinkon, Gbegamey, Saint Jean, Vodje for a total of 09 corn samples and 06 popcorn samples in this zone.
- Zone 3 (West sector): comprising Tokpa, Akpakpa, Abattoir, Degakon, Souroulere for a total of 16 corn samples and 09 in this zone.

At each sampling point, a uniform sample mass of 250 g was collected in sterile polyethylene bags using a random selection procedure from vendors' available stock. All collected samples were immediately transported to the laboratory in insulated coolers at 4˚C and stored under dark, dry condition at −20˚C to halt any ongoing fungal metabolism or toxin production prior analyses.




Figure 1. Sampling.

### 2.2. Moisture Content

Moisture content of corn and popcorn were determined according to [14] by heating at 105˚C for 2 h to constant weight.

## 2.3. Mycological Analysis

Fungal contamination was evaluated by the direct seeding method for popcorn maize and indirect method for popcorn (see Section 2.3.1). The morphological identification of the fungal species was made according to the method described by [15]. All media used in this study were from Oxoid, Ltd, made in the United Kingdom.

The toxinogenic nature of the fungal strains was determined by molecular biology (see below) associated with a determination of the secondary metabolites by Thin Layer Chromatography (TLC) according to [16].

### 2.3.1. Determination of Mycoflora

Fungal contamination was evaluated using distinct protocols tailored to each matrix type. For corn kernels, a direct seeding was applied: four (04) intact grains were surface-sanitized in a 5% sodium hypochlorite (bleach) bath for 1 minute, rinsed three times with sterile distilled water according to the modified Usler method [17], and transferred onto Potato Dextrose Agar (PDA) plates. For finished popcorn, an indirect method was employed: samples were finely ground, and 5 mg portion of the resulting flour was suspended in 10 mL o 0.1% sterile peptone water. A 100 µL aliquot of this suspension was spread-plated onto potatoes dextrose agar (PDA). All plated matrices were incubated in the dark at 25˚C for 5 to 7 days.

### 2.3.2. Enumeration

Colony appearing on primary PDA plates were quantified to determine isolation frequency and relative density. Presumptive fungal genera were isolated and subcultured onto specialized differential media, including Malt Extract Agar (MEA), Yeast Extract Sucrose (YES) agar, and *Aspergillus flavus parasiticus* agar (AFPA). Identification of *Aspergillus* section *Flavi* and other molds was performed at the species level based on macro-morphological features (colony, color, texture) and microscopic characteristic (condiophore structure, vesicle shape, phialide arrangement, conidia surface) using standard taxonomic keys.

### 2.3.3. Species Frequency and Relative Density

The prevalence and distribution of the fungal flora were expressed mathematically. The isolation frequency (*Fq*) represents the percentage of total analyzed samples (*N*) that contained a specific fungal group, calculated as follow:

Equation (1) is: $Fq = \left(ns/N\right) \times 100$

where: *ns* = Number of samples with a genus or species, *N* = Total number of samp1es.

The relative density (*R*) represents the proportion of isolates belonging to a specific fongal group out of the total number of fungal isolates obtained across the entire matrix group, calculated as follow:

Equation (2) is: $R\left(\%\right) = \left(n_i/N_i\right) \times 100$

where: $n_i$ = Number of isolates of a genus or species, $N_i$ = Total number of fungi isolated.

The toxigenic nature of *Aspergillus* section *Flavi* was evaluated by PCR of some of the aflatoxin genes and thin layer chromatography after extraction with chloroform.

## 2.4. Molecular Characterization

### 2.4.1. DNA Extraction

Each isolate was grown on Potatoes dextrose agar (PDA) for 5 days in the dark at 25˚C. Mycelia were collected and DNA was extracted with phenol-choloroform according to Aamir [18]. Total DNA was quantified and visualized by gel electrophoresis. In addition, PCR amplifications of the $\beta$-tubulin region were used to verify the quality of the genomic DNA extractions for the *Aspergillus* genus.

### 2.4.2. Polymerase Chain Reaction

PCR was performed according to [17]. Five clustered genes for aflatoxin production (AflO-AFLA_139000, AflD-AFLA_139110, AflP-AFLA_138990, AflQ-AFLA_138980 and AflR-AFLA_139220) were amplified. The primers were used at a concentration of 100 pmol/µl, DNA template, 50 ng, Taq Ozyme Purple mix 2, 25 µl for a total volume of 50 µl. PCR conditions wereas follows: denaturation at 95˚C for 5 min; 40 cycles of 94˚C for 45 seconds, primer-specific annealing temperature (at 60˚C for $\beta$ tubulin and 58˚C for all other) for 1 min, and extension at 72˚C for 1 min; and a final extension at 72˚C for 10 min. The reactions were performed in GenAmp PCR 2700 thermocycler (Applied Biosystems, Forster City, CA, USA). To ensure assay validity, *Aspergillus flavus* NRRL 3357 DNA was utilized as a positive amplicon control, while a No Template Control containing sterile PCR-grade water served as negative control. PCR products were resolved by electrophoresis on a 1.5% agarose gel. Amplification was scored positive as positive when fluorescent bands matched the expected amplicon size relative a molecular marker and according the reference *Aspergillus flavus* NRRL 3357, and negative in the complete absence of a band.

PCR primers used are described by [17] and shown in **Table 1**.

**Table 1.** Primers for PCR.

| Genes | Nucleotides |
|---|---|
| b-tubulin control | Btub1F: GTTCACCTTCAGACCGGCCAGTG<br>Btub1R: CTCCTTCATGGAGACCTTTCCGCG |
| b-tubulin 2 (RFLP) | Bt2a: GGTAACCAAATCGGTGCTGCTTTC<br>Bt2b: ACCCTCAGTGTAGTGACCCTTGGC |
| AflD | AflDF: CGGTGTATTTGGTCACCGGGGC<br>AflDR: CGGCTGCCTGGGCATCAGTTTC |
| AflQ | AflQF: CGTTATGGGAGGATCGGACACG<br>AflQR: CCCAGATCTGATCCTCCTGCG |

**Continued**

| | |
|---|---|
| AflP | AflPF: GGGCATTCATGCCTTGGTTG<br>AflPR: CCCATACCTAGATCAAAGCGG |
| AflO | AflOF: CTCTGGCGAAGGTCGGCATTG<br>AflOR: CTCTCGGCCAGGAAGTCAGG |
| AflR | AflRF: GTCGATTTCTTGGCCGAGTC<br>AflRR: CTCAGCAAGTAGCCATCCTG |

#### 2.4.3. Restriction Fragment Length Polymorphism (RFLP)

The RFLP of a segment of $\beta$-tubulin was determined to identify the clade of species of *Aspergillus* section *Flavi* isolated. After PCR of $\beta$ tubulin partial sequence, 20µL of product are digested by BstYI: *A. flavus* (336, 438, 581bp); *A. parasiticus* (176, 263, 334, 576bp).

### 2.5. Mycotoxins Extraction and Analysis

Aflatoxins extraction from corn and popcorn matrices were realized and analyzed according to a modified liquid-liquid partitioning protocol [19]. A 25 g ground analytical portion was extracted with 125 mL of a methanol/water mixture (85:15, v/v) by vigorous mechanical shaking for 3 minutes, then filtered through Whatman No. 1 paper. A 100 mL aliquot of the filtrate was amended with 100 mL of 10% sodium chloride (NaCl) aqueous solution to minimize emulsions. The mixture was transferred to a separatory funnel and washed with 50 mL of hexane; the upper hexane layer containing lipids was discarded while the lower aqueous-methanolic phase was retained. Aflatoxins were then partitioned from the aqueous phase by adding 125 mL of chloroform. Following separation, the upper aqueous phase was discarded, and the lower chloroform phase was collected. The extract obtained was filtered through Whatman No. 1 containing 2.5 g of anhydrous sodium thiosulfate to eliminate residual water. The dried chloroform extract was evaporated to dryness under reduced pressure using with a rotary evaporator, and the residue was completely dried at 60˚C. The final dry extract was reconstituted in 50 µL of pure methanol and stored at 4˚C in amber vials until analysis.

To screen the toxigenic profile of the isolated fungal strain each isolate was grown on Yeast Extract Sucrose agar for 7 days at 27˚C. Half of the agar plate was ground in chloroform. The mixture was shaken by vigorous mechanical shaking for 3 minutes and filtered through Whatman No. 1 paper. The solvent was evaporated and the mixture of mycotoxin were re-dissolved in methanol. Aspergillic acid production was screened on AFPA medium using a ferric chloride colorimetric assay, targeting the characteristic orange-brown reverse coloration.

Analysis of extracts obtained were performed by Thin Layer Chromatography according to [16]. 10 µL of extracts and standards of aflatoxins B1, B2, G1 and G2 (Sigma Aldrich) are spotted onto the fluorescent chromatographic plate (1.05553. DC-Alufolien Kieselgel 60). Migration solvent was ether/methanol/water (96: 3: 1, v/v/v). The plates were placed in the solvent system, dried and observed at UV

365 nm.

## 3. Results

### 3.1. Moisture Content

The moisture content of raw corn kernels ranged from 26.0% to 28.0% with a mean value of 27.3%. Popcorn samples exhibited an identical mean moisture content of 27.6%, with observed individual sample values distributed within a narrow range from 27.2% to 28.0%. This high moisture retention in both raw and processed street-vended commodities indicate storage and display environments that strongly support fungal growth.

### 3.2. Microbiology Analysis

Fungal contamination was prevalent across all analyzed commodities. In raw corn kernels, *Aspergillus* section *Flavi* was the most frequent contaminant (*Fq* = 30.0%), followed by *Aspergillus* section *Nigri* (*Fq* = 25.0%), *Mucorales* (*Fq* = 10.0%), and *Penicillium* spp. (*Fq* = 5.0%). Conversely, in finished popcorn samples, *Aspergillus* section *Nigri* was the most widespread group, exhibiting an isolation frequency of 61.5% (detected in 16 out of 26 samples), followed by section *Flavi* (*Fq* = 38.5%; 10/26), *Penicillium* spp. (*Fq* = 26.9%; 7/26), and *Mucorales* (*Fq* = 15.4%; 4/26). The distribution metrics, comprising Isolation Frequency (*Fq*) and Relative Density (R) calculated exclusively out of the total isolated fungal colonies per commodity group, are structured in Table 2.

Table 2. Fungal contamination.

| | *Aspergillus section Nigri* | *Aspergillus section Flavi* | *Penicillium* spp. | *Mucorales* |
|---|---|---|---|---|
| **Corn** | *Fq* = 25%<br>R = 48.7% | *Fq* = 30%<br>R = 34.1% | *Fq* = 5%<br>R = 4.9% | *Fq* = 10%<br>R = 12.3% |
| **Popcorn** | *Fq* = 61.5%<br>R = 41.3% | *Fq* = 38.5%<br>R = 33.3% | *Fq* = 26.9%<br>R = 14.7% | *Fq* = 15.5%<br>R = 10.7% |

Note: *Fq* (Isolation Frequency) represents the percentage of samples positive for the genus/section; R (Relative Density) represents the percentage of total isolates collected within that specific commodity category. Total strains of Aspergillus section Flavi successfully isolated and purified for subsequent characterization numbered 45 (14 from corn kernels and 31 from popcorn).

#### 3.2.1. *In Vitro* Toxigenic Potential and Molecular Profile of *Aspergillus* Section *Flavi* Strains

A total of 45 isolates belonging to *Aspergillus* section *Flavi* were subjected to in vitro profiling to assess metabolic and biosynthetic capacity. Ferric chloride assays confirmed that all 45 isolates produced aspergillic acid. TLC screening of the culture extracts revealed that 44 out of the 45 isolates possessed functional capabilities to produce aflatoxins, with variations in specific profiles: 29 isolates (9 from corn, 20 from popcorn) secreted the full spectrum of aflatoxins (B1, B2, G1, and

G2), 10 isolates produced B1 and G1, and 5 isolates synthesized B1 alone. One isolate recovered from popcorn was found to be a non-producer under the specified culture conditions, displaying a non-aflatoxigenic chemical phenotype (Table 3).

Table 3. Aflatoxins production by isolated strains.

| | Numbers of strains according to aflatoxins production profile | | | | | |
|---|---|---|---|---|---|---|
| | B1 only | B1 & B2 | B1 & G1 | B1, B2 &G1 | B1, B2, G1 & G2 | Not producer |
| **Corn** | 0 | 0 | 5 | 0 | 9 | 0 |
| **Popcorn** | 5 | 0 | 5 | 0 | 20 | 1 |

PCR amplifications demonstrated that 97.8% (44/45) of the isolates successfully carried all five targeted structural and regulatory genes (aflD, aflO, aflP, aflQ, and aflR) from the aflatoxin cluster, showing perfect alignment between genetic biosynthetic potential and observed phenotypic toxin production. Subsequent β-tubulin RFLP analysis following BstYI enzymatic digestion revealed that 100% of the corn isolates (14/14) and 80.65% of the popcorn isolates (25/31) exhibited the restriction fragment pattern diagnostic of the *Aspergillus parasiticus* clade, while the remaining 6 popcorn isolates clustered with the *Aspergillus flavus* clade (Table 4).

Table 4. Strains distribution by *Aspergillus parasiticus* and *flavus* clade.

| | *Aspergillus parasiticus* clade | *Aspergillus flavus* clade |
|---|---|---|
| **Corn** | 14 | 0 |
| **Popcorn** | 25 | 06 |
| **Total** | 39 | 06 |

### 3.2.2. Direct Occurrence of Aflatoxins in Marketed Samples

Direct analysis of the food items sampled from commercial channels revealed extensive natural mycotoxin contamination. Aflatoxins were detected in 61.5% (16 out of 26) of the street-vended finished popcorn samples. For raw corn kernels, the contamination incidence was 37.5%, with 15 out of 40 analyzed market samples testing positive for aflatoxins.

## 4. Discussion

Popcorn is increasingly recognized as both a nutritionally beneficial and economically accessible food product [20]. Its palatability has contributed to its growing popularity among the Beninese population, particularly among children consumption is increasingly integrated into the dietary habits of the Beninese population, particularly among children. However, the safety of this product with respect to mycotoxin contamination remain a public health concern.

The present study establishes a high baseline incidence of aflatoxins in raw corn

kernels (37.5%; 15/40) and an even higher occurrence in street-vended finished popcorn (61.5%; 16/26). This significant presence of mycotoxins in the final product is directly associated with the poor quality of the raw material. Maize is an exceptionally favorable substrate for the colonization of aflatoxigenic molds, particularly members of *Aspergillus* section *Flavi*, when environmental parameters are unmitigated [21] [22]. The high moisture contents observed across our sample frame (means of 27.3% and 27.6%) significantly exceed safe storage thresholds (typically below 13% - 14%), creating a permissive macro-environment for mold proliferation and active secondary metabolism.

Mycological data indicate a notable distribution of aflatoxigenic aspergilli. Molecular RFLP screening establishing that a substantial majority of the section *Flavi* isolates belong to the *Aspergillus parasiticus* clade rather than *Aspergillus flavus*. This finding is consistent with specific regional ecologies described in international literature [23] [24]; for instance, [25] observed a localized predominance of *A. parasiticus* in agricultural maize fields in France, demonstrating that under distinct microclimatic and substrate conditions, this highly toxigenic species becomes the primary driver of multi-aflatoxin (B and G groups) accumulation. The high incidence in finished popcorn (61.5%) matches international trends where informal or inadequately controlled corn processing lines fail to eliminate mycotoxin loads. [13] reported significant aflatoxin contamination in popcorn kernels marketed in Spain, and [26] found persistent mycotoxin residues in processed corn snacks. The global threat is further highlighted by the 2018 European Rapid Alert System for Food and Feed (RASFF) notification regarding high levels of aflatoxins and ochratoxin A in commercial popcorn within the Luxembourg food network [27], reinforcing that standard heat processing is an insufficient safeguard.

The high rate of contamination in finished popcorn relative to raw kernels illustrates the thermodynamic stability of these compounds. Popcorn production relies on rapid heating of kernels to expand internal moisture until the outer pericarp ruptures [28]. While this structural transformation involves temperatures that induce starch puffing, chemical degradation of aflatoxins requires sustained temperatures ranging between 237˚C and 306˚C [29], which are rarely reached uniformly throughout the short popping cycle. Although popping can achieve partial reductions of initial toxin burdens by approximately 50% to 70% via mechanical and thermal decomposition [30], the excessive initial mold and mycotoxin load present in poorly stored Beninese raw corn ensures that the residual concentration in the finished street food remains high. This creates a chronic exposure risk for pediatric consumers, highlighting an urgent need for regulatory interventions.

## 5. Conclusion

This study demonstrates that people are exposed to health risks through the consumption of popcorn that may be contaminated with aflatoxins. Multiple strains

of aflatoxin-producing *Aspergillus* from the *Flavi* section were isolated from both corn kernels and popcorn. Given that children are the primary consumers of this snack food in Benin, it is crucial to implement a quality control system specifically targeting street-vended popcorn products intended for this vulnerable population.

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## Author Contributions

Conceptualization Yann C.S. Adjovi; methodology, Yann C.S. Adjovi and Ulrich Hilarion Ahehehinnou; software, X Yann C.S. Adjovi.; validation, Yann C.S. Adjovi; formal analysis, Yann C.S. Adjovi and Ulrich Hilarion Ahehehinnou; investigation, Yann C.S. Adjovi and Ulrich Hilarion Ahehehinnou; resources, Yann C.S. Adjovi; data curation, Yann C.S. Adjovi and Ulrich Hilarion Ahehehinnou; writing—original draft preparation, Yann C.S. Adjovi; writing—review and editing, Ulrich Hilarion Ahehehinnou; visualization, Yann C.S. Adjovi; supervision, Yann C.S. Adjovi; project administration, Yann C.S. Adjovi; funding acquisition, Yann C.S. Adjovi. All authors have read and agreed to the published version of the manuscript.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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