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Characterization of Fungal Contaminants in Fermented Locust Beans in Kwara State, Nigeria: Implications for Food Safety

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Abstract

Fermented locust bean seeds are utilized for seasoning in various traditional dishes. Its production is mostly under unhygienic conditions by majorly illiterate women who care less about microbial contamination of the final product. Consumption of any contaminated food can cause health complications in humans. This study was aimed at isolating the mycoflora associated with fermented locust bean seeds collected from three villages in Kwara State, Nigeria using both morphological and molecular techniques. DNA of each of the isolates was extracted using Zymo Fungal/Bacteria DNA Miniprep Kit. PCR amplification of the ITS regions of the isolates was carried out using primer pair; ITS1 and ITS4. The products had been sequenced, and the results from the BLAST search revealed that Isolate A was *Aspergillus flavus*, Isolate B was *A. niger*, Isolate C was *Rhizopus arrhizus*, and Isolate D was *Mucor indicus*. The fermented locust bean seeds from Madi village had the highest fungal count (7.2 X 10^3 cfu/gm), while that from Ogundele village had the lowest (1.4 X 10^3 cfu/gm). The presence of *Aspergillus niger* and *A. flavus* (mycotoxigenic fungi) in this study poses a significant public health risk. Therefore, it is important to encourage the villagers involved in fermented locust bean production and storage in these villages to be more hygienic.

Keywords: Fermented locust beans seeds, fungal contamination, molecular techniques, morphological, public health risk

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Introduction

The perennial tree known scientifically as *Parkia biglobosa*, commonly called the locust beans plant, belongs to the Fabaceae family [1]. Found predominantly in tropical areas of Africa, notably in nations like Nigeria, Ghana, Senegal, and Cameroon, this tree is referred to as the African locust bean in Nigeria. It holds notable significance in various African cultures for both culinary and medicinal applications. Parkia tree is a moderate-sized plant that grows to a maximum height of 20 meters, producing extended pods characterized by a rich, mahogany-colored exterior [2]. The pods, measuring around 30 centimeters in length, encase numerous flat, oval-shaped seeds with a dark brown color [3]. The seeds pass through a fermentation process to become condiment used in food seasoning in numerous traditional African recipes because of their aromatic quality and unique flavor [4].

Known as "dawadawa (Hausa)," "Ogiri (Igbo)," or "iru (Yoruba)", the 3 major languages in Nigeria, fermented locust bean seeds have diverse health benefits. Adding them to recipes introduces a range of flavors, enhancing the holistic sensory experience of the food [5, 6]. Fermented locust beans enhance substantial protein content, dietary fiber, as well as provide B-complex vitamins, along with nutrients like calcium, iron, and magnesium [7, 8]. They encompass bioactive elements like octadecanoic acid and n-hexadecanoic acid, known for their antioxidant and antimicrobial attributes [9, 10]. In addition, fermented locust beans stimulate the proliferation of lactic acid bacteria, such as *Lactobacillus* spp, fostering improved gut health, increased nutrient absorption, and fortification of the immune system. Consuming fermented locust beans also contributes to regulating blood sugar levels, among other health benefits [11,12], promoting the multiplication of dermal fibroblasts thereby contributing to the process of wound healing [13], activity against malaria-causing parasites, Protecting humans from bacterial diseases [14] and addressing and managing coccidiosis in poultry birds [15].

Poor processing and storage procedures due to the low levels of education and standard of living of majority of the women involved in large scale production of fermented locust beans seeds can compromise their quality and safety for human consumption [16]. Fungi, such as *Aspergillus* spp. and *Fusarium* spp. may proliferate in poorly stored fermented locust beans seeds, leading to mycotoxins secretion. Mycotoxins, such as aflatoxins, ochratoxins and fumonisins are carcinogenic, hepatotoxic, nephrotoxic, and possess immunosuppressive properties [17]. This raises concerns regarding the potential ingestion of fungi and possibly their mycotoxins in improperly processed fermented locust beans seeds. It is, therefore, pertinent to investigate the fungal species associated with fermented locust bean seeds so as to provide valuable

insights into the extent of fungal contamination and the associated risk(s).

Materials and Methods

Study area

The survey was carried out in three different villages known for large scale production and sales of fermented locust beans seeds in Kwara State, Nigeria namely Madi village (8°29'23.3"N, 4°26'59.3"E), Aiyede village (8°29'24.0"N, 4°27'07.0"E.) and Ogundele village (8°29'24.8"N, 4°28'07.0"E). One kilogram of healthy fermented locust bean seeds, aged 3-5 days, was purchased from the villages and stored in labeled zip-lock bags. They were transported to the Unilorin Biology laboratory for analysis on the same day.

Fungal isolation and morphological identification

Potato Dextrose Agar (PDA) culture medium was prepared according to the manufacturer's instruction. Dilution plate technique as described by Akharenegbe *et al.* [18] was adopted for the fungal isolation of the study. A test tube containing one gram of fermented locust beans was mixed with 9 ml of sterile distilled water. The solution of fermented locust beans seeds was subjected to successive ten-fold dilutions until reaching a concentration of 10^{-3} [19].

Twenty milliliters of sterilized molten PDA was cooled to 45°C and then poured in Petri dishes containing a 1 ml aliquot of the serially diluted sample (10^{-3}), which was dispensed in triplicate. Each plate was gently swirled and allowed to solidify. The plates were incubated at 28°C for 72 h. The fungal colonies on each plate were enumerated and inoculated onto fresh PDA for subculture to obtain pure cultures of the isolates. The morphological characteristic of the mycelium including the macroscopic and microscopic features of each isolate were noted with reference to Kidd *et al.* [20]. Percentage occurrence of fungal species was calculated using the formula:

$$\text{Percentage (\%) occurrence of fungal species} = \frac{\text{Number of fungal species}}{\text{Total number of fungi isolated}} \times 100$$

The obtained values were subjected to analysis of variance.

Pure cultures of fungi were stored in refrigerator at 4°C prior to DNA extraction.

Molecular identification of the different fungal isolates

Quick-DNA ™ Fungal Mini Prep Kit (Zymo Research Group, California, and USA) was used to extract Genomic DNA of the isolated fungi. The protocol of the above named kit was used. Fungal mycelium from each isolate was scrapped off the culture plate using a sterilized surgical blade and transferred to a sterilized mortar. Next, 750 µl of bashing bead buffer was introduced to the sample, and homogenization was carried out using liquid nitrogen. The resulting homogenate was then transferred to an Eppendorf tube and subjected to centrifugation as directed by the protocol. Subsequent steps including cell lysis, precipitation and DNA purification were executed according to the manufacturer's instructions. Pure DNA was stored at 4°C for further analysis.

The extracted DNA was sent to Inqaba biotec, Ibadan, Nigeria, for genetic amplification and sequencing in both directions using the Internal Transcribed Spacer 1 (ITS1) with the DNA pattern TCCGTAGGTGAACCTGCGG and ITS4 with the sequence TCCTCCGCTTATTGATATGC. Sequencing was conducted using ABI 3500 genetic analyzer (Thermo Fisher Scientific, Massachusetts, USA). Sanger sequencing utilized a DNA primer, single-stranded DNA template, deoxynucleotide triphosphates (dNTPs), DNA polymerase and di-deoxynucleotide triphosphates (ddNTPs). Incorporation of ddNTP at intervals terminated DNA chain elongation, halting the extension process.

With the "forward" and "reverse" genomic patterns obtained after sequencing, alignment was performed using BioEdit v. 7.2.5 software to generate consensus sequences. Subsequently, a comparison between the isolates' sequences and those available in the National Center for Biotechnology Information (NCBI) database and Global Biodiversity Information Facility (GBIF) Database was conducted utilizing the BLAST tool.

Results and Discussions

The study focused on the fungal species associated with fermented locust beans produced in three villages in Kwara State, Nigeria known for high level of fermented locust bean production for sale. The fungal counts of the fermented locust beans seeds analyzed in this study varied between 7.2 X 10^3 and 1.4 X 10^3 cfu/gm on the average, with Madi village exhibiting the highest count (7.2 X 10^3 cfu/gm) and Ogundele village recording the lowest count (1.4 X 10^3 cfu/gm) (Table 1). The results align with some reported results. For example, Okolo *et al.* [21] in their study on fungal contamination in fermented locust beans seeds, reported diverse fungal counts in the different markets from which the samples were obtained, with certain markets displaying elevated contamination levels than others (2.0 × 10^5 CFU/g to 4.33 × 10^5 CFU/g). The high fungal count observed in Madi village in contrast to the other villages in this study may be due to the low level of sanitation there.

Table 1: Average fungal counts of the plates of fermented locust bean seeds

Villages	Fungal count (× 10^3cfu/gm)
Madi	7.10 ± 0.10^a
Aiyede	2.20 ± 0.10^b
Ogundele	1.00 ± 0.10^b

Mean with same letter(s) within columns are not significantly different ($P \leq 0.05$)

Four fungal species were isolated from the locust bean seeds. They were initially labeled as Isolates A to D, which were morphologically identified tentatively as *Aspergillus flavus* (Fig. 1a and b), *A. niger* (Figs. 2a and b), *Rhizopus arrhizus* (Figs. 3a and b) and *Mucor* sp. (Figs. 4a and b) based on their observed macroscopic and microscopic morphological features (Table 2).

Table 2: Morphological identification of the fungal isolates

Fungal Isolates	A	B	C	D
Macroscopic Characters	Yellowish-green	Dense layer of black colour	White cottony mycelia that transitions to brown.	Cottony to fluffy white colonies
Microscopic Characters	Conidiophores originate from a basal cell. Vesicles shaped like clubs are located at apex of the conidiophores.	Slender conidiophores bearing brown spherical vesicles adorned with black pigmented spore heads	Sporangiospores are formed, by columella at the apex of the sporangiophore.	Erect sporangiophores are hyaline and branched, featuring both long and shorter branches.
Tentative Identity	*Aspergillus flavus*	*A. niger*	*Rhizopus arrhizus*	*Mucor* sp.

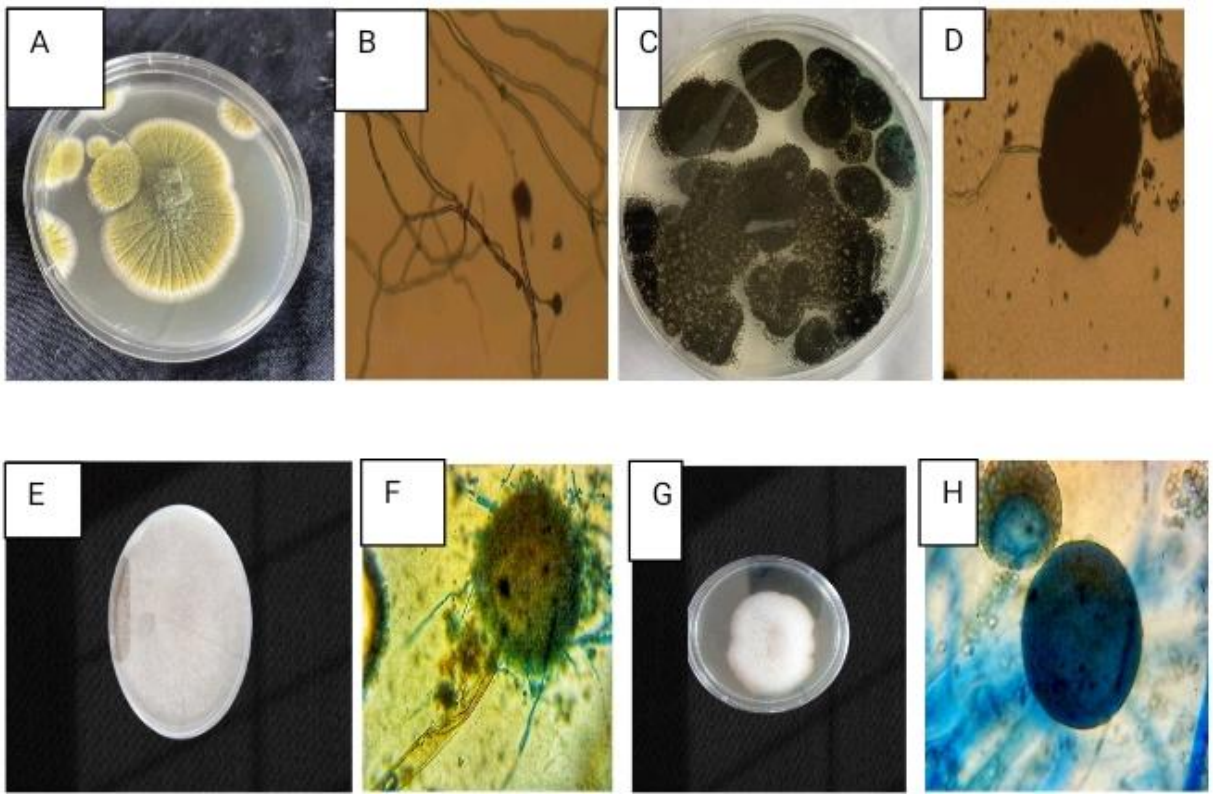

Plate 1: A- Culture of Isolate A on PDA; B- Micrograph of Isolate A; C- Culture of Isolate B on PDA; D- Micrograph of Isolate B; E- Culture of Isolate C on PDA; F- Micrograph of Isolate C; G- Culture of Isolate D on PDA; H- Micrograph of Isolate D

Molecular characterization was done to confirm the identity of the isolates. The DNA sequence of the ITS regions were 567, 555, 597 and 599 bp for isolates A to D respectively. Table 3 displays the BLAST-n search results in the GenBank using these ITS sequences revealing the percent identity, query cover and the matched accession number of the DNA sequences obtained from each of the fungal isolates. None of the individual query cover and percentage identity was less than 98 and 97%, respectively in this study, thereby authenticating their identities. According to Raja *et al*. [22], Ajadi and Olahan [23], dependable identification through molecular techniques involves achieving a query cover of at least 80% and a percentage identity of at least 97% in the comparison of isolated fungal species sequences with those in the NCBI database, utilizing the Basic Local Alignment Search Tool for Nucleotides (BLASTN) Sequences. Use of molecular markers such as ITS and others is very popular nowadays for correct identification of fungal species [24, 25].

Table 3: Species identified through BLAST searches of the DNA sequences

Isolates	Organisms (Accession numbers)	Query Cover	Percent Identity (%)
1	*Aspergillus flavus* (KX067890.1)	97	100
2	*Aspergillus niger* (MW600264.1)	89	100
3	*Rhizopus arrhizus* (OR536571.1)	100	99.66
4	*Mucorindicus* (JN561265.1)	98	98.48

All the fungal species reported to contaminate fermented locust beans in this study agreed with results of some previous studies. Nwadiaro *et al.* [26] in their study on fungi associated with stored locust bean in Jos, Plateau State, reported the presence of *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., *Curvularia* sp., *Mucor* sp. and *Fusarium* sp. Yayaha *et al.* [27] also isolated *Aspergillus* sp., *Candida albican*, *Botrytis* sp., *Penicillium* sp. and *Curvularia* sp. from locust beans available for purchase in the markets of Wudil and Darki in Kano State, northwestern region of Nigeria. Similarly, Oyewole *et al.* [28] isolated *Aspergillus niger*, *A. fumigatus*, *A. flavus*, *A. terrus*, *Saccharomyces cerevisiae*, *Mucor* sp. and *Penicillium* sp. from some local spices including locust beans sold in Minna, Nigeria. Okolo *et al.* [21] isolated *Aspergillus niger*, *Rhizopus arrhizus*, *Fusarium cladosporium*, *F. cummunis*, *Aspergillus nudilans*, *Aspergillus tamari* and *Aspergillus terreus* from fermented locust beans collected from Lokoja, Kogi State.

Number of isolates per fungal species and per village is shown in Table 4. The identified fungi occurred most in the fermented locust beans seeds purchased from Madi village (51.72%), while fermented locust beans seeds purchased from Ogundele village had the lowest fungi occurrence (20.69%) in this study.

Table 4: Number of isolates per fungal species and per village

Villages	*Aspergillus flavus* (n)	*A. niger* (n)	*Rhizopus arrhizus* (n)	*Mucor indicus* (n)
Madi	4.00 ± 2.00^{b}	9.00 ± 1.00^{a}	2.00 ± 1.73^{bc}	0.00 ± 0.00^{c}
Aiyede	4.00 ± 1.73^{a}	0.00 ± 0.00^{b}	4.00 ± 1.00^{a}	0.00 ± 0.00^{b}
Ogundele	0.00 ± 0.00^{c}	4.00 ± 0.00^{a}	1.00 ± 0.00^{b}	1.00 ± 1.00^{b}

Where n = number of fungal species

Table 5: Percentage fungal species distribution in the fermented locust beans

Fungal species	No of Isolates	Percentage occurrence (%)
Aspergillus flavus	8 ± 0.816^{b}	27.59
A. Niger	13 ± 0.816^{a}	44.83
Rhizopus arrhizus	7 ± 2.16^{b}	24.14
Mucor indicus	1 ± 0.816^{c}	3.45

Percentage fungal species distribution in the fermented locust beans is presented in Table 5. *Aspergillus niger* occurred most frequently (44.83%), while *Mucor indicus* had the lowest fungal species distribution (3.45%). The results agreed with the work of Nwadiaro *et al.* [26] and Okolo *et al.* [21] who reported that *Aspergillus niger* had the highest rate of occurrence in fermented locust beans seeds in their respective studies. They separately concluded that *Aspergillus niger* may be the major organism responsible for spoilage of fermented locust bean seeds. In contrast to the above, Adekoya *et al.* [29] in their study on fermented foods in Nigeria reported *Aspergillus flavus* as the most dominant fungi associated with fermented foods in Nigeria.

Aspergillus niger, *Mucor indicus* and *Rhizopus arrhizus*, play a positive role in food fermentation, contributing to the development of desirable flavors and textures [30]. Some species of *Aspergillus* can potentially produce mycotoxins which can contaminate food and pose health risks to humans and livestock [31, 32, 33].

Conclusion

Aspergillus flavus, *A. niger*, *Rhizopus arrhizus* and *Mucor indicus* were isolated from fermented locust beans seeds purchased in 3 villages known for large scale production of fermented locust beans seeds in this study. These fungal species can contaminate food and pose health risks to humans and livestock. The findings highlight the critical need for strict hygiene and sanitation practices in the processing and storage of fermented locust beans to reduce fungal growth and mycotoxin production.

Conflict of interest: There is no conflict of interest among the authors.

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