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Molecular Characterization of Multi-Drug Resistant Histamine-Producing Gram-Negative Bacteria Isolated from Fermented *Prosopis africana* Seeds (*okpee*) in Nigeria

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ABSTRACT

Background: Fermented *Prosopis africana* seeds (*okpee*) are naturally protein-rich but exude odors that attract houseflies. These flies can carry contaminants, including pathogens and histamine-producing bacteria. Bacterial histamine in food can cause headaches and pseudo-hypersensitivity reactions. This study investigated multidrug-resistant, histamine-producing Gram-negative bacteria (GNB) isolated from *okpee* sold in Enugu Ezike, Nigeria.

Methods: Histamine content of the mesquite seed samples was determined using high performance liquid chromatography. Bacteriological isolates from randomly sampled *okpee* were identified by 16S rRNA gene sequencing. Antibiotic susceptibility of the isolated bacteria was assessed using the Kirby-Bauer disk agar diffusion method. The isolates were also screened for resistant plasmids and virulence genes (*Stx2* and *toxA*).

Results: Histamine levels of the fermented samples averaged 1.0×10^{-4}-6.2×10^{-1} mg/kg. Of 240 samples of *okpee* screened, 25 (10.5%) were contaminated with histamine-producing GNB, including *Pseudomonas* spp., *Escherichia coli*, *E. fergusonii*, and *Shigella flexneri*. All the isolated histamine-producing bacteria were resistant to amoxicillin, erythromycin, ampicillin, and cefuroxime, with more than 50% exhibiting resistance to the other antibiotics tested. The test isolates were found to habor multiple resistant plasmids (size: 0.5 kbp-10 kbp) and virulence factor genes *Stx2* and *toxA*.

Conclusion: This study observed a high prevalence of bacterial contaminants capable of producing histamine in o*kpee* sold in Enugu Ezike, Nigeria. The isolates were multidrug-resistant and harbored virulent genes, posing a serious public health risk. Therefore, strengthening hygiene practices during the production and handling of o*kpee* is necessary to prevent contamination and ensure product safety.
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Keywords: Fermentation, Gram-negative bacteria, Histamine, Virulence factors

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1. INTRODUCTION

In Nigeria and many other African countries, traditionally fermented foods provide an alternative source of protein and contribute to food security. One such condiment, known locally as '*okpee'* by the Enugu-Ezike people, is made from fermented mesquite seeds (*Prosopis africana*), which are protein-rich and obtained from the pods of the mesquite tree[1-3]. Currently, many African countries, especially Nigeria, are facing serious economic challenges that have worsened the food shortage due to skyrocketing inflation, poor supply chains, and limited access to food, especially proteinous

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foods[4,5]. The use of leguminous seeds, such as mesquite seeds, provides an alternative source of protein, especially for rural dwellers[6]. The seeds are rich in many amino acids, with histidine being the most abundant[7]. Research has shown that fermentation enhances the total amino acid content of *okpee*[8].

Many varied bacteria have been isolated from *okpee*[9], and most of them have been found to ferment *P. africana* seeds[10]. Some of these bacterial fermenters produce and release exogenous histamine into their environment. Histamine-producing bacteria can generate histamine in fish products, fermented foods, and beverages because they produce the histidine decarboxylase enzyme, which catalyzes the conversion of histidine to histamine[11]. The presence of histidine induces the transcription of histidine decarboxylase genes[12]. Previously, numerous bacteria have been reported to produce exogenous histamine in fermented food products and palm wine[13-15].

Histamine is a toxic biogenic amine that exerts its effects on the functions of various body tissues and systems, especially in individuals with histamine intolerance[16]. This intolerance causes gastrointestinal accumulation of histamine due to a deficiency in diamine oxidase, which subsequently leads to increased absorption into the bloodstream[17]. As a result, histamine accumulates in the vascular system, thereby causing syndromes such as persistent headaches, respiratory allergic disease, skin and soft tissue allergies, menstrual disorders, and altered intestinal function[18-20]. Consumers of *okpee* cannot detect this histidine derivative because it is colorless and odorless[21,22]. Hence, ingesting large amounts of histamine during meals is possible, which may lead to histamine intoxication.

Presently, Africans living in rural areas such as villages in Enugu-Ezike are faced with diverse health challenges such as poor health promotion orchestrated by the lack of proper hygiene maintenance, unsafe handling and storage of food items, self-medication, and misdiagnosis. In these areas, it is common practice to prepare food materials, such as processing mesquite seed with impure water, which grossly contaminates the seeds. Furthermore, the way this natural product is stored, handled, and sold in the open environment of the rural markets encourages microbial contamination. In the area, it is common to observe some consumers of this product manifest ill health ranging from nausea to severe gastroenteritis after consumption of food prepared with this *okpee*; however, the exact cause remains unknown. In view of the foregoing, it is essential to find out whether toxic substances are released by bacterial contaminants in this food condiment, locally called *okpee*, especially during fermentation and storage. The goal of this study was to investigate the prevalence of multidrug-resistant, histamine-producing Gram-negative bacteria (GNB) present in the fermented mesquite seeds in Enugu-Ezike, Nigeria.

2. MATERIALS AND METHODS

2.1. Study area

The test samples were purchased from the rural markets in Enugu-Ezike (Nigeria), one of the largest towns in the Nsukka cultural zone. Maps showing the study area can be found in Figure 1.

2.2. Culture media and antibiotic discs

Both the culture media and antibiotic discs were bought from Oxoid (Germany (England). The artificial culture media were eosin methylene blue agar, Salmonella-Shigella agar, nutrient agar, MacConkey agar, Mueller-Hinton agar, Brain heart infusion broth (BHIB), and Niven agar. The standard antibacterial discs used were ceftriaxone (30 µg), amoxicillin (30 µg), ampicillin (30 µg), clindamycin (10 µg), erythromycin (15 µg), gentamicin (30 µg), ciprofloxacin (10 µg), chloramphenicol (10 µg), ofloxacin (10 µg), tetracycline (30 µg), and pefloxacin (10 µg).

2.3. Test samples and sample collection

The test samples were fermented and unfermented seeds of *P. africana.* The pods, unfermented seeds, and fermented seeds (*okpee*) can be found in Figure S1, respectively. Two hundred and sixty test samples, comprising 240 *okpee* (fermented seeds of *P. africana*) and 20 unfermented samples, were purchased randomly from different markets in Enugu-Ezike. The samples were kept inside sterile universal containers and stored at refrigeration temperature until microbiological analysis and histamine studies. The samples were bought from the various markets in the study area, which serve Enugu-Ezike indigenes as well as people from the neighboring villages within the Nsukka zone in the trading of foodstuff.

2.4. Screening of '*okpee*' for histamine production using high-performance liquid chromatography (HPLC)

HPLC was employed to quantitatively assay for the presence of histamine in both fermented and unfermented mesquite seeds. This technique was conducted to confirm the role of fermentation and bacterial fermenters in histamine biosynthesis.

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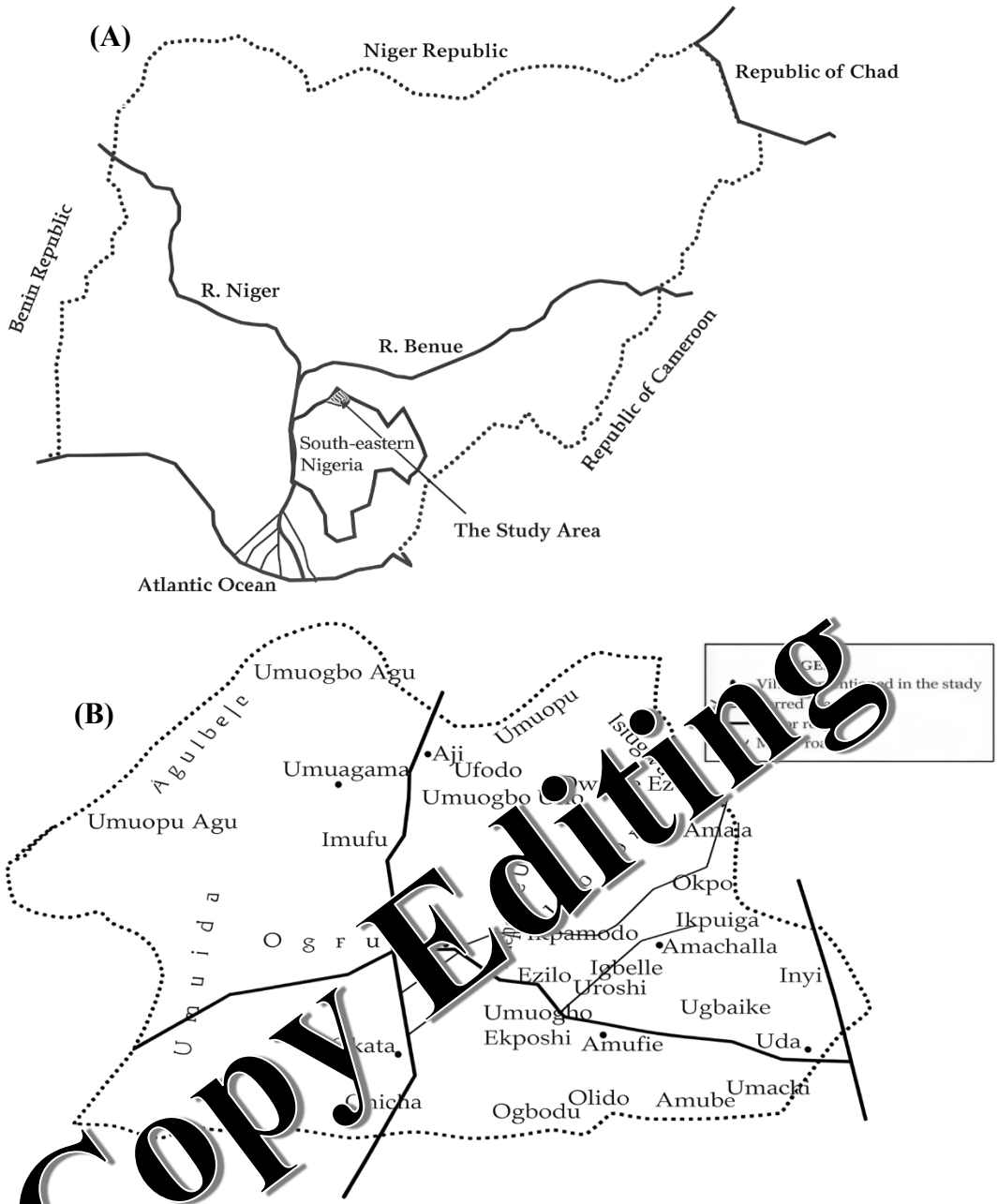

Fig. 1. (A) Niger[illegible]map showing the study area; (B) Map of Enugu-Ezike, showing all the villages where the samples were bought. Adapted from "Rura[illegible]" accessed 16th March, 2025)[23].

2.5. Extraction of histamine from fermented and unfermented samples

A total of 50 samples, 10 per group, arranged based on the duration of fermentation, were randomly selected to quantify the histamine content. Extraction and analysis of histamine were performed by modification of the previously described techniques[24]. About 20 mg of the triturated sample was transferred to a 1,000 mL volumetric flask, and 5% trichloroacetic acid was added to the volumetric flask mark for dilution. The mixture was mixed for 1.5 minutes and sonicated for 5 minutes. From this solution, 250 mL was collected and diluted to 4.5 mL in a volumetric flask with methanol:water (50:50). One milliliter of the resulting solution was collected and filtered with a membrane filter of size 0.45 µL.

2.6. Derivatization of ortho phthalaldehyde for HPLC analysis

A 100 mL of the derivatization agent–ortho phthalaldehyde was collected from the reagent vial and injected into the samples. The mixture was vortexed,

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and the reaction was allowed to complete in 5 minutes. A microsyringe was thoroughly washed and used to pipette 5 mL of the derivatized samples into the HPLC column.

2.7. Separation of histamine by HPLC

The HPLC system (the Agilent 1200 Series) was set up and allowed to stabilize for 30 minutes. A 5 µL of each derivatized sample was introduced into the system at a flow rate of 1.0 mL/min. The conditions for running HPLC were maintained as follows: column (chromsep SS C18 measuring (150 mm × 4.6 mm × 5 µm), mobile phase consisting of mobile phase A (tetrahydrofuran: methanol:phosphate-buffer [100 mmol/L; 1:8:9]), and mobile phase B (methanol:phosphate-buffer; 100 mmol/L; 80:20), with gradient program of min/A%B%: (8/75/25), (12/67/33), (25/50/50), (30/0/100), and (35/67/33). The filtration of mobile phase was carried out through a membrane filter of pore size 0.4 µm and degassed. Histamine was quantified using a detector (AGILENT 1260) at a wavelength of 254 nm.

2.8. Microbiological analysis

2.8.1. Isolation of GNB

Five hundred milligrams of each sample was introduced into a tube containing peptone water (1% of 0.1%) and incubated at 37°C for 12–16 h for homogenization. Then, 1.0 mL of each homogenized sample was mixed with 4 mL of tube of BHIB and incubated at 37°C overnight for enrichment. A loopful of the culture in BHIB was inoculated in duplicate onto MacConkey agar, cetrimide agar, Salmonella-Shigella agar and eosin methylene blue, and then incubated at 37°C overnight. At 24 h of incubation, all organisms/ colonies cultured on the culture media were individually subjected to the Gram-staining technique. All the GNB were further characterized using standard microbiological methods, and the identity of each strain was confirmed by the amplification and sequencing of the 16S rRNA gene.

2.8.2. Isolation of histamine producers

Each pure strain of genetically confirmed isolates was subcultured onto duplicate plates of Niven's agar and incubated under aerobic conditions at 37°C for 72 h. Purple colonies were indicative of histamine production[25]. The histamine producers were subcultured onto a nutrient agar slant, incubated at 37°C overnight, and then maintained at refrigeration temperature for future use.

2.9. Strain-specific confirmation by DNA sequencing

2.9.1. Genomic DNA extraction

DNA extraction was carried out using the ZR Fungal/Bacterial DNA MiniPrep™ (50 preps; model D6005; Zymo Research, California, USA). Two milliliters of the broth culture of the test bacterial isolate was introduced into a ZR Bashing™ Lysis Tube, and 750 µL of Llysis solution was added. The tube was secured in a bead-fitted 2 mL tube holder assembly and processed at maximum speed for 10 minutes. The lysis tube was then spun at 12,000 ×g for 60 seconds. A 450 µL aliquot of the supernatant was transferred to a Zymo-SpinTM IV Spin Filter placed in a collection tube and centrifuged at 7,000 ×g for 60 seconds. Then, a 1,2L00 µL of binding buffer was added to the filtrate in the collection tube. The buffer-filtrate mixture (800 µL) was transferred to a Zymo-SpinTM IIC column in a collection tube and spun at 10,000 ×g for 60 seconds. The flow-through was discarded from the collection tube, and the mixture was centrifuged again. A 200 µL aliquot of DNA pre-wash buffer was added to the Zymo-Spin™ IIC column placed in a new collection tube, followed by centrifugation at 10,000 ×g for 60 seconds. Then, 500 µL of Fungal/Bacterial DNA washing buffer was transferred to the column and centrifuged at 10,000 ×g for 60 seconds. The Zymo-Spin™ IIC column was added to a clean 1.5 mL microcentrifuge tube, and 100 µL of DNA elution buffer was added directly to the column matrix. It was centrifuged at 10,000 ×g for 30 seconds to elute the DNA. The resulting ultra-pure filtrate (DNA) was used as the template for subsequent analysis.

2.9.2. 16S rRNA gene amplification and sequencing

The test bacterial strains were identified by the amplification and sequencing of the 16S rRNA gene. The specific primers were designed by the authors and supplied by Inqaba Biotech Limited (Inqaba Biotechnical Industries Ltd., South Africa). The polymerase chain reaction (PCR) mix was composed of 12.5 µL of Taq 2× Master Mix (New England Biolabs; M0270, USA), 1 µL each of 10 µM forward (27F: AGAGTTTGATCMTGGCTCAG) and reverse (1525R: AAGGAGGTGWTCCARCCGCA) primers, 2 µL of DNA template, and 8.5 µL of nuclease-free water. The cycling conditions used for the amplification of the *16SrRNA* gene were: initial denaturation at 94°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 56˚C for 30 seconds, and elongation at 72°C for 45 seconds. This process was followed by a final elongation step at 72°C for 7 minutes and a hold temperature at 10°C indefinitely. The amplified fragments were sequenced using a Genetic Analyzer 3130xl (Applied Biosystems, Thermo Fisher Scientific, Tokyo, Japan) following the manufacturer's manual. The sequencing kit used was the BigDye Terminator v3.1 Cycle Sequencing Kit. BioEdit

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software and MEGA X were used for all genetic analyses.

2.10. Screening for virulence factor genes

2.10.1. Gene amplification of the bacterial strains

The PCR mix consisted of 12.5 µL of Taq 2× Master Mix from New England Biolabs (M0270), 1 µL each of 10 µM forward (Stx2 F:CCGGAATGCAAATCAGTC and ToxA F: CTGCGCGGGTCTATGTGCC) and reverse (Stx2R: CAGTGACAAAACGCAGAACT and ToxA R: GATGCTGGACGGGTCGAG) primers, 2 µL of DNA template, and 8.5 µL of nuclease-free water.

2.10.2. Cycling conditions for the amplification of *Stx2* gene

The order of the conditions used was as follows: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 45 seconds. This process was followed by a final elongation step at 72°C for 7 minutes and a hold temperature of 10°C indefinitely.

2.10.3. Cycling conditions for the amplification of *toxA* gene

The conditions used consisted of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 45 seconds. This process was followed by a final elongation step at 72°C for 7 minutes and a hold temperature at 10°C indefinitely.

2.11. Plasmid analysis of the test bacteria

2.11.1. Plasmid DNA extraction

Plasmid analysis was carried out using Zyppy™ Plasmid miniprep kit (catalog no. D4036). Bacterial culture (600 µL) grown in LB medium was added to a 1.5 mL microcentrifuge tube, centrifuged at 16,000 ×g for 30 seconds, and the supernatant was discarded. Then, 100 µL of 7X lysis buffer (Blue) was added to the sediment, and the tube was mixed by inverting six times. The formation of a clear blue color indicated complete lysis. Next, 350 µL of cold neutralization buffer (Yellow) was added and mixed thoroughly. The sample turned yellow, followed by the formation of a yellowish precipitate when the neutralization was complete. The sample was re-centrifuged at 11,000–16,000 ×g for 2-4 minutes, and the supernatant (~900 µL) was transferred into the provided Zymo-Spin™ IIN column. The column was placed into a collection tube and centrifuged for 15 seconds. The flow-through was discarded, and the column was placed back into the same collection tube. Then, 200 µlL of Endo-Wash buffer was added to the column and centrifuged for 30 seconds. Subsequently, 400 µL of Zyppy™ washing buffer was added to the column and centrifuged for one minute. The column was transferred into a clean 1.5 mL microcentrifuge tube, and 30 µL of Zyppy™ elution buffer was added directly to the column matrix. The tube was allowed to stand for one minute at room temperature, followed by centrifugation for 30 seconds to elute the plasmid DNA.

2.11.2. Plasmid profiling by agarose gel electrophoresis

Two grams of agarose were measured out and mixed with 100 mL of 1X TAE buffer in a microwavable flask. The mixture was gently heated by microwaving for three minutes until the agarose had completely dissolved. The agarose solution was allowed to cool to about 50°C (i.e., a safe temperature at which the flask can be comfortably held in the hand) for 5 minutes. Then, 10 µL of EZ-Vision DNA stain was added to allow the stain to bind to the DNA, enabling visualization of the DNA under ultraviolet light. The agarose was poured into a gel tray with the well comb in place. The newly poured gel was kept at 4°C for 15 minutes until it was completely solidified. Loading buffer was mixed with each of the purified plasmid DNA samples. The agarose gel was placed into the gel box, and a molecular weight ladder was loaded into the first lane of the gel. The samples were carefully loaded into the additional wells, and the gel was run at 100 V for 60 seconds. After running, the power was turned off, the gel was removed, and the purified plasmid fragments were visualized.

2.12. Test for antibiotic sensitivity

Genetically confirmed strains of GNB were subcultured on nutrient agar for 16–24 h at 37°C to obtain cultures at the exponential phase of growth. Then, the colonies were standardized by matching the turbidity of each isolate with that of 0.5 McFarland opacity standard. The standardized colonies were subjected to an agar diffusion test using selected antibiotic discs (Oxoid, UK) representing the antibiotics used in the treatment of bacterial infections in the study area. The discs used included ceftriaxone (30 µg), amoxicillin (30 µg), ampicillin (30 µg), clindamycin (10 µg), erythromycin (15 µg), gentamicin (30 µg), ciprofloxacin (10 µg), chloramphenicol (10 µg), ofloxacin (10 µg), tetracycline (30 µg), and pefloxacin (10 µg). All the plates were kept on the bench at room temperature for 30 minutes and then incubated at 37°C overnight. The inhibition zone diameters per disc per plate were measured. The sensitivities of the isolates were determined using the guidelines of the Clinical Laboratory Standards Institute (CLSI), version 2023[26].

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Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the reference strains for the antibiotic susceptibility studies.

2.13. Multiple antibiotic resistance index (MARI)

MARI was determined by calculation using the formula: MARI = $^{a}/_{b}$, where "a" is the number of antibiotics to which the test isolates were resistant, and "b" is the total number of antibiotics against which it was evaluated.

2.14. Data analysis

Data obtained from this study were analyzed and evaluated on the basis of means and percentage values. Tables and Figures were used (where appropriate) for the presentation of results. Statistically, a descriptive analysis was performed, and variables were analyzed with the Statistical Package for Social Sciences (SPSS) version 23.0 (SPSS; Chicago, IL, USA). Differences in data were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Histamine content of the *P. africana* seeds

The fermented and unfermented (control) samples subjected to HPLC analysis revealed the presence and absence of histamine, respectively. Table 1 shows the average quantity of histamine in the fermented samples as obtained by HPLC-enhanced integrator analysis. No histamine was detected in any of the unfermented samples. The average histamine content per group (mg/kg) of fermented *okpee* ranged from 1.0×10^{-4} to 6.2×10^{-1}. At $p < 0.05$, the histamine content values showed a significant difference in the intragroup analysis of *okpee*, while there was no significant difference between each *okpee* in the intergroup analysis.

3.2. Characterization of GNB

Of 240 fermented samples of *okpee* screened, 25 (10.5%) were found to be contaminated with histamine-producing GNB. The strains identified belonged to the genera *Pseudomonas, Escherichia*, and *Shigella* spp. They included *P. aeruginosa SYLI*, *P. gessardi*i K2(1), *P. putida* BC-4, *P. putida P.* Putida_19_1031, *E. coli* K_EC180, *E. fergusonii* APO3, and *Shigella flexneri* ATCC 29903, with the respective accession numbers PQ220167, PQ220165, PQ220295, PQ220296, PQ220300, PQ220162, ON075468, and PQ220162 (Table 2).

3.3. Gene encoding virulence factors in the isolated histamine-producing GNB

Figures 2 and 3 are the gel electrophoresis images showing the amplification of the genes *toxA* and *stx2* in the test isolates. The *toxA* gene, which encodes exotoxin A, was found in the test *Pseudomonas* spp., and *the stx2* gene, which encodes Shiga toxin, was detected in *E. coli*, *E. fergusonii*, and *S. flexneri*.

3.4. Plasmid analysis of the [illegible] histamine-producing GNB

Gel electrophoresis of the plasmid DNA extracted from the test isolates revealed the presence of plasmids ranging in size from 0.5 kbp to 10 kbp. The profile is shown in the gel image of Figure 4. All the isolates were found to harbor at least one plasmid of size 10,000 bp. Strains with laboratory codes A and D, which represent *P. aeruginosa* and *E. coli*, respectively, had varied plasmids ranging from 600 bp to 10 kbp. Strains C and E representing *P. putida* and *S. flexneri,* had plasmid DNA sizes from 500 bp to 10,000 bp. Code B, representing *E. Fergusonii*, had one plasmid of size 10,000 bp.

3.5. Antibiotic sensitivity pattern of the histamine-producing GNB

The results of the antibiotic sensitivity test on the isolates are shown in Table 3. All the test isolates (100%) showed resistance to erythromycin, amoxicillin, ampicillin, and cefuroxime. More than 50% of the tested *Pseudomonas, Escherichia*, and *Shigella* species exhibited resistance to more than 60%, 90%, and ب70% of all tested antibiotics, respectively. Apart from gentamicin, which showed good activity against the tested *Pseudomonas* spp., other tested antibiotics was not potent against up to 50% of the bacteria studied. Table 4 shows the sensitivity-resistance profile and MARI of the isolates studied. All the histamine

Table 1. Histamine content of the fermented *P. africana* seeds obtained by HPLC enhanced integrator analysis

Group of test samples based on the duration of fermentation	No of samples per group	Average quantity per group (mg/kg)	Mean amount produced (mg/kg)
Control group (unfermented samples)	10	0.00	0.00
Group 1 (2 days)	10	1.0×10^{-4}	3.7×10^{-1}
Group 2 (4 days)	10	3.5×10^{-1}	
Group 3 (6 days)	10	5.2×10^{-1}	
Group 4 (8 days)	10	6.2×10^{-1}	

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Table 2. Molecular identification of the isolated HP GNB by DNA sequencing

Sample ID	Scientific name	Strain	Max score	Total score	Query cover (%)	E value	Per. ident (%)	Accession number
1	*P. aeruginosa*	SYLI	1764	1764	100	0	99.48	PQ220167
2	*P. gessardii*	K2(1)	1393	1393	99	0	96.68	PQ220165
3	*P. putida*	BC-4	2002	2002	100	0	98.93	PQ220295
4	*P. putida*	*Pseudomonas Putida*_19_1031	1356	1356	99	0	95.23	PQ220296
5	*E coli*	K_EC180	1323	9207	99	0	95.15	PQ220300
6	*E. fergusonii*	APO3	1353	1853	99	0	96.30	ON075468
7	*S. flexneri*	ATCC 29903	1474	1474	99	0	99.75	PQ220162

Max: maximum; E value: expectation value in BLAST

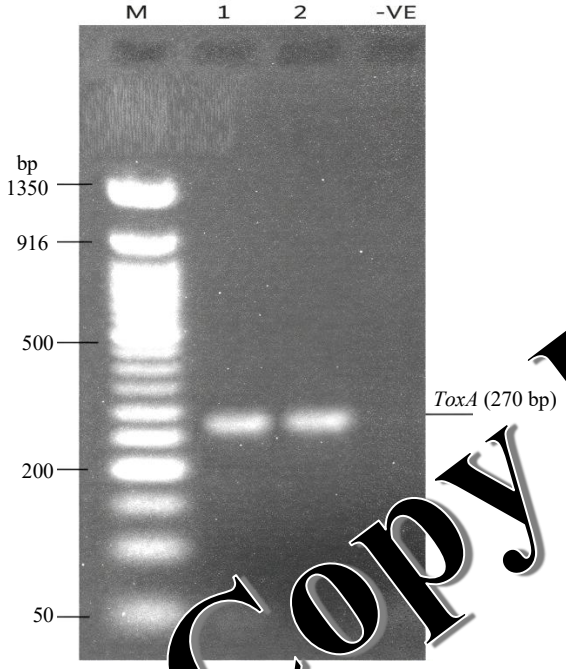

Fig. 2. Gel image showing the amplification of the *ToxA* gene at 270 bp. Lane 1: *P. gessardii*; lane 2: *P. putida*; lane -VE: negative no template control; lane M: 50 bp DNA ladder.

producers were resistant to amoxicillin and erythromycin. Ampicillin, and cefuroxime, showing that they are multidrug-resistant superbugs. Some isolates were found to be extensively drug-resistant, while one (*E. coli* K_EC180) was pan-drug resistant. This study recorded MARI values ranging from 0.64 to 1.0.

4. DISCUSSION

In Africa, the traditional fermentation of African mesquite seeds generates dark-brown fermented *P. africana* seeds called *okpee*. Fermentation promotes the nutritional value of foods, prolongs their shelf life, provides an alternative use for neglected crops, and provides employment opportunities for many producers of *okpee*[27]. However, the process results in the formation of secondary metabolites such as histamine, which causes various symptoms observed in histamine poisoning and histamine intolerance, or both[28,29].

The results of this study revealed the presence and absence of histamine in all the fermented and unfermented *P. africana* seeds, respectively. This observation strongly indicates that histamine is released by microorganisms during the fermentation of food, which is rich in amino acid, histidine[30]. The presence of a measurable amount of histamine in the first group (two days of fermentation) showed that histamine

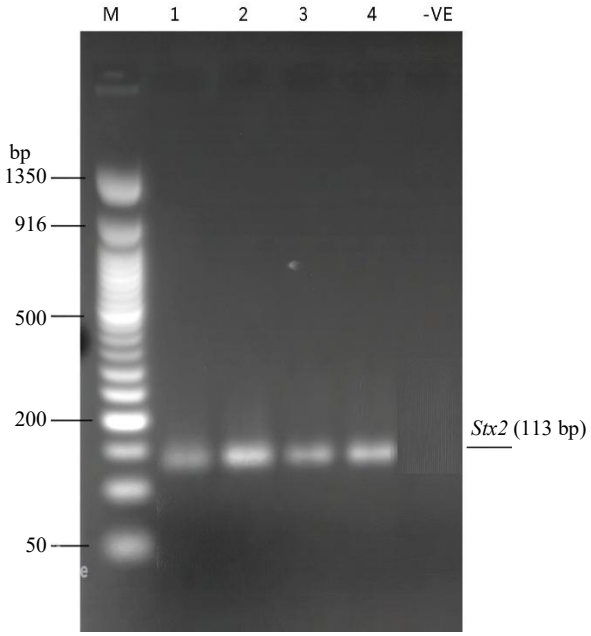

Fig. 3. Gel image showing the amplification of the *stx2* gene at 113 bp. Lane 1: *S. flexneri*; lane 2: *E. fergusonii*; lanes 3 and 4: *E. coli*; lane -VE: negative template control; lane M: 50 bp DNA ladder.

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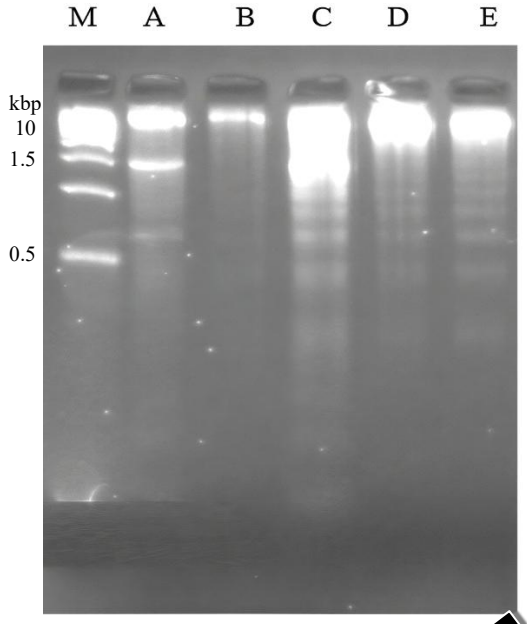

Fig. 4. Electrophoresis gel image showing plasmid profiles ranging from 0.5 kbp to 10 kbp. Isolates A and D have plasmids ranging from 600 bp to 10 kbp, while isolate B has only 10 kbp plasmid. Isolates C, D, and E have plasmids ranging from 0.6 bp to 10 kbp. Lane A: *P. aeruginosa*; lane B: *P. putida*; lane C: *E. coli*; lane D: *E. fergusoni*; lane E: *Shigella flexneri*; lane M: 1 kbp DNA ladder.

production by contaminating bacteria immediately after the commencement of fermentation processes. The decarboxylation of histidine in *okpee* is of great significance to consumer health, especially in individuals with histamine non-hypersensitivity pseudoallergy, in which a small blood concentration of histamine can trigger many observable adverse reactions affecting multiple body systems[31]. It has been reported that histamine accumulation of more than 40 mg/meal or 0.75 mg/kg body weight is considered hazardous[32]. In the present study, the range of average histamine content (mg/kg) of *okpee* was 1.0×10^{-4} to 6.2×10^{-1}. Although this level is too low to cause histamine poisoning in general consumers, it is high enough to cause idiosyncratic reactions in individuals with histamine intolerance. Due to its palatable taste and incorporation into many dietary staples, this fermented product is consumed daily in many parts of Nigeria. Consequently, the frequent consumption of the food product containing *okpee* results in blood histamine accumulation in individuals with diamine oxidase deficiency, leading to health challenges such as urticaria pigmentosa, migraine, gastroenteritis, and cardiovascular maladies[31]. Within each group of samples analyzed (intragroup analysis), there was no statistically significant difference between histamine contents obtained at the same fermentation time. Conversely, there was a statistically significant difference among the histamine contents of the samples analyzed across different fermentation periods (intergroup analysis), highlighting the critical role of fermentation duration in histamine accumulation, which increased with time, as shown in Table 1. This observation is consistent with previous research[33]. The reason for higher amounts of histamine in the seeds fermented for six days might be due to high levels of microbial contamination, which increased the fermentation rate by these organisms. Therefore, histamine poisoning or histamine intolerance may be suspected if a set of unexpected syndromes occurs in different ways after ingestion of food or soups prepared

Table 3. Antibiotic susceptibility pattern of the histamine-producing GNB (n = 25)

S/n	Ant	S (%)	Ps I (%)	R (%)	S (%)	Es I (%)	R (%)	S (%)	Sf I (%)	R (%)
1	ERY	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	25(100)	0 (0)	0 (0)	25 (100)
2	AMX	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	25 (100)
3	CH	5 (20)	0 (0)	20 (80)	1 (4)	1 (4)	23 (92)	10 (40)	0 (0)	20 (80)
4	PEF	10 (40)	5 (20)	10 (40)	10 (40)	2 (8)	13 (52)	10 (40)	5 (20)	10 (40)
5	TET	1 (4)	1 (4)	23 (92)	1 (4)	1 (4)	23 (92)	3 (12)	0 (0)	22 (88)
6	CPX	12 (48)	1 (4)	12 (48)	10 (40)	3 (12)	12 (48)	10 (40)	0 (0)	15 (60)
7	GN	20 (80)	0 (0)	5 (20)	10 (40)	0 (0)	15 (60)	12 (48)	1 (4)	12 (48)
8	OFX	5 (20)	0 (0)	20 (80)	5 (20)	0 (0)	20 (80)	5 (20)	0 (0)	20 (80)
9	CN	12 (48)	1 (4)	12 (48)	6 (24)	0 (0)	19 (76)	12 (48)	1 (4)	12 (48)
10	AMP	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	25 (100)
11	CEF	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	25 (100)	0 (0)	0 (0)	25 (100)

Ant: antibiotics; ERY: erythromycin; AMX: amoxicillin; CH: chloramphenicol; PEF, pefloxacin; TET: tetracycline; CPX: ciprofloxacin; GN: gentamicin; OFX: ofloxacin; CN: clindamycin; AMP: ampicillin; CEF: ceftriaxone; Ps: *Pseudomonas* species; Es, *Escherichia* species; Sf, *S. Flexneri,* S, sensitive; I, intermediately sensitive; R, Resistant.

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Table 4. Susceptibility resistance profile and MAR index of the representative Gram-negative histamine producers

Strains tested	Sensitive to	Resistant to	MARI
P. aeruginosa SYLI	PEF, CPX, CTO, GN	AMX, CN, AMP, OFX, ERY, CH, TET	0.64
P. gessardii K2 (1)	PEF, CPX, CTO, GN	AMX, CN, AMP, OFX, ERY, CH, TET	0.64
P. putida BC-4	OFX, GN, CN	AMX, PEF, AMP, ERY, CH, TET, CPX, CTO	0.73
P. putida 19_1031	PEF, CPX, TET	AMX, CLN, AMP, ERY, CH, CTO, OFX	0.73
E. coli K_EC180	NIL	CTO, GN, AMX, PEF, CLN, AMP OFX, ERY, CH, TET, CPX. CTO	1.0
E. Fergusonii APO3	PEF CTO	AMX, PEF, CLN, AMP OFX, ERY, CH, TET, CPX, MP, CTO, GN	0.82
S. flexneri ATCC 29903	PEF, CPX, CTO, GN	AMX, CN, AMP OFX, ERY, CH, TET	0.64

PEF, pefloxacin; CPX: ciprofloxacin; CTO: ……; GN: gentamicin; OFX: ofloxacin; CN: clindamycin; TET: tetracycline; NIL: ….; AMX: amoxicillin; AMP: ….; ERY: erythromycin; CH: ….; CHc: chlorampenicol GN: ; AMP: ampicillin; CEF: ceftriaxone.

using fermented mesquite seeds. Such presentations call for serious public health concern, because some of the invading organisms, such as the GNB under study, have the propensity to produce exogenous histamine and, more significantly, occupy a leading position in the causation of different infections[34,35].

The present study determined the prevalence of multidrug-resistant histamine-producing GNB that contaminate *okpee* during and after fermentation, which was about 10.5%. Given the microbial population in this condiment, the high isolation rate of histamine-producing GNB identifies them as significant fermenters, which is consistent with previous studies[36]. The organisms genetically identified in the study include *P. aeruginosa SYLI*, *P. gessardii* K2(1), *P. putida* BC-4, *P. putida P*. 19_1031, *E. coli* K_EC180, *E. fergusonii* AP and *S. flexneri* ATCC 29903 (Table 2). This corresponds several GNB isolates from fermented *P. africana* seeds, which is in agreement with the results of similar investigations conducted in Makurdi (Northern Nigeria) by Ekhuemelo and Iordye, who isolated *Shigella* spp. and other germs from *P. africana* condiment sold in their markets[37]. Similarly, Agunwah et al. isolated *E. fergusonii* and other bacteria from fermented African mesquite seeds in Anambra State, Nigeria[9]. Moreover, Ogbu et al. isolated *E. coli* from fermented mesquite seed sold in Abuja, Nigeria[38]. Such bacterial contaminants may have been deposited on the condiments by houseflies or other flies, which land on these fermented products and contaminate them with organisms transferred from their bodies or feces. In addition, bacteria from non-sterile plant leaves, containers, and equipment used for fermentation, as well as from mesquite seed handlers, may contaminate *okpee* during the process. Contamination of the fermented product by any of the GNB isolated is a serious public health threat because each of the bacteria has been implicated in various nosocomial or non-hospital infections[39-41].

P. aeruginosa and *E. coli* isolated in this study, belong to an important group of pathogens called ESKAPE group, which have a significant impact on health care-associated infections as they can resist the biocidal activity of antibiotics. Furthermore, all the test isolates (100%) were resistant to erythromycin, amoxicillin, ampicillin, and ceftriaxone, showing that they are multidrug-resistant bacteria. Also, each test organism was susceptible to only a few antibiotics, which is consistent with other research reporting that strains of GNB are becoming increasingly insensitive to several antibiotics[42]. The antibiotic resistance profile of the GNB obtained in this study showed that some of the bacteria are multidrug-resistant, extensively drug-resistant, or pan drug-resistant, which is in agreement with the results obtained elsewhere[43]. In addition, our study reported high multiple antibiotic resistance indices, perhaps due to consistent exposure of bacteria to antibiotics during treatment, which allows for antibiotic selection pressure. Furthermore, the presence of resistant plasmids (size: 0.5 kbp-10 kbp) might have contributed to the multiple antibiotic resistance observed in these isolates.

The present study examined the presence of some virulence factor genes that could contribute to the pathogenicity of these foodborne pathogens. The *toxA* gene, which encodes exotoxin A, was found in the tested *Pseudomonas* spp., and *the stx2* gene, which encodes Shiga toxin, was detected in *E. coli*, *E fergusonii*, and *S .flexneri.* The *toxA* gene encoding the major virulence factor in *P. aeruginosa* was detected by other researchers in some bacteria isolated from some clinical specimens[44], which is consistent with our findings. *Stx*, detected in this study, has been found to be the main virulence factor in Shiga toxin-producing *E. coli*[45]. Shiga toxins, the ribosome-inactivating proteins, are produced by *Shigella* spp. as well as *E. coli* because both organisms can harbour the *stx2 gene*[46], which is in agreement with our findings. Both toxins (*toxA and stx2)* are host 'cell destroyers'; thus, the presence of bacteria that release them in *okpee* is harmful to public health. This observation is important because their presence in bacteria isolated from the food

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material indicates a high risk to food safety, primarily due to their connection with severe human illnesses. The *stx2* increases the risk of developing hemorrhagic colitis and hemolytic uremic syndrome, a condition that causes kidney failure. Again, inappropriate use of antibiotics can induce phage-mediated expression of *stx2*, leading to increased toxin production and thus making treatment seriously challenging[47]. Apart from inhibiting protein synthesis and causing tissue necrosis, the *toxA* gene often co-exists with biofilm-associated genes in contaminated food samples, intensifying the bacteria's survival and persistence in food processing environments[48]. The presence of these virulence genes underscores the necessity for strict hygiene standards and proper cooking conditions to eliminate the bacteria.

There may be some potential limitations in this study. The key one is the lack of information on the method(s) used in preparing the *okpee* samples, as both the fermented and unfermented samples were bought randomly from different markets in Enugu-Ezike without taking cognizance of how they were processed and fermented. Future research should clearly group the *okpee* samples according to their method of production/fermentation in order to study, differentiate, and compare various *okpee* microbiota. Additionally, a small study area with a small sample size may be a possible limitation of the findings of this study. Future researchers should, therefore, use larger sample sizes collected from many states in Nigeria and different geographical regions to better understand the relationship between environmental factors and histamine contents of the fermented *okpee.* Furthermore, future research should consider the effects of different storage conditions on the microbial quality and histamine content of *okpee*, as our findings could also be seen in the light of such a limitation.

5. CONCLUSION

This study observed high prevalence of GNB contaminants with the capability to produce histamine in *okpee* sold in Enugu Ezike, Nigeria. The isolates were multidrug resistant and also harbored virulent genes that could constitute a serious public health menace due to their direct contribution to life-threatening diseases. Therefore, significant improvements in hygienic practices during production and handling are necessary to prevent product contamination and ensure safety.

DECLARATION

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Generative AI and AI-assisted technologies

No generative AI technology was used in the production of this manuscript.

Ethical approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

AC, US, AF, EV, UT, OG, OH, and IE: sample collection, data collection and analysis. AC and US: manuscript writing.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

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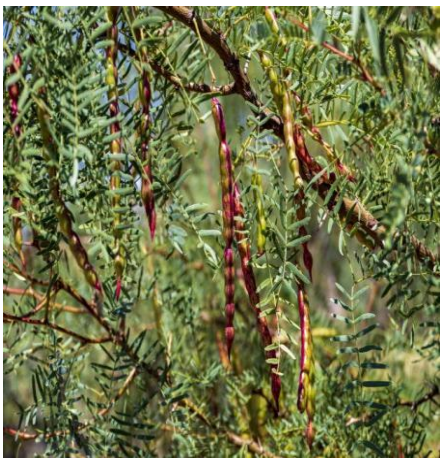

Fig. S1. (A) Mesquite tree showing the pod; (B) fermented seed (*okpee*); (C) mesquite seeds (unfermented seeds).

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