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Research article

Microbiological evaluation of the indigenous fermented condiment *okpeye* available at various retail markets in the south-eastern region of Nigeria

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ABSTRACT

In Africa, indigenous fermented condiments contribute to food security as a low-cost source of protein. *Okpeye* is an indigenous fermented condiment produced from *Prosopis africana* seeds. The reliance on spontaneous fermentation processes and unhygienic practices during production often results in the contamination of the final product with microbial hazards. A microbiological evaluation of 18 commercial samples of *okpeye* purchased from six markets in two cities in southeastern Nigeria was conducted. Fifty-nine (59) bacteria were isolated and identified at the species level by phenotyping and sequencing the 16S *rRNA*, *gyrB* and *rpoB* genes. *Bacillus* (47.4 %) and *Staphylococcus* (42.3 %) were the predominant bacterial genera in *okpeye*. Overall, *B. amyloliquefaciens* and *S. simulans* were the most frequently occurring bacteria and were present in all samples. In addition, *B. cereus* was isolated in samples obtained from all markets. Other bacterial species included *B. velezensis, Oceanobacillus caeni*, *S. cohnii, Escherichia fergusonni* and *Vagacoccus lutrae.* The *B. cereus* isolates (10) were screened for the presence of 8 enterotoxin genes (*hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *cytK*, *entFM*) and one emetic gene (*cesB*). The non-haemolytic enterotoxin (*nheABC*) and haemolytic enterotoxin (*hblABD*) complexes were present in 70 % and 50 % of *B. cereus* respectively. The positive rate of *cytK* and *entFM* genes was 70 %, while the *cesB* gene was 30 %. Antibiotic susceptibility assessment showed that most of the isolates were susceptible to gentamicin, tetracycline, streptomycin, and erythromycin but resistant to ciprofloxacin and vancomycin. These findings highlight the need for further controls to reduce contamination with potential pathogenic bacteria in indigenous fermented condiments such as *okpeye*. There is also a need to educate producers regarding hygienic practices to safeguard public health and food security.

1. Introduction

Okpeye (also referred to as *okpehe, kpaye*) is a spontaneously alkaline fermented condiment obtained from *Prosopis africana* (mesquite) seeds popularly consumed in the Southeast and North Central regions of Nigeria [1,2]. Although the traditional role of

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condiments is to enhance flavour, they are also a source of nutrients and may contain biologically active ingredients which may offer health benefits [3]. Indigenous condiments are rich in protein and serve as an inexpensive alternative to animal-based protein sources [4,5].

Okpeye is predominantly prepared by small-scale producers at home and sold in local markets [6]. Traditional production involves sorting and boiling mesquite seeds until soft. Seeds are then dehulled manually using fingertips and reheated until any excess moisture is removed. The dehulled seeds are transferred into a basket lined and covered with leaves, locally referred to as *okpeye* leaves. The covered seeds are usually placed under the sun for 6–8 days to allow fermentation to occur. Fermented seeds are then moulded into shape and allowed to dry completely to give a dark, pungent-smelling product known as *okpeye* (Fig. 1.) [7].

Bacillus spp. are reported to be the most important species involved in the fermentation of several indigenous African fermented condiments. These include *soumbala*, *iru*, *daddawa* and *ogiri* using raw materials such as locust beans and melon seeds [8–10]. The biochemical activities of *Bacillus* during fermentation are also responsible for the acceptable organoleptic characteristics such as taste, flavour, visual appearance, and texture [11,12] associated with these foods. However, microbial hazards may be introduced from contaminated raw materials, water utensils and the fermentation environment. The uncontrolled nature of the production process and limited awareness and use of hygienic practices for food preparation and storage lead to inconsistencies in the microbial profile of each fermentation batch. This compromises the microbiological quality and safety of many indigenous fermented foods [13]. Consequently, foodborne pathogenic bacteria are routinely isolated from plant-based fermented condiments available for commercial sale giving rise to food safety and public health concerns [14,15].

In Africa, indigenous fermented foods make an immense contribution to food security [16]. Fermentation improves the nutritional value of foods, extends shelf life, provides an alternative use for underutilized crops, and provides a source of income for many small-scale producers [17]. However, the presence of microbial hazards presents a significant hurdle to their utilization. This is particularly important as the potent antimicrobial effects of low pH and acid production that are characteristic of lactic fermented foods are usually absent in fermented condiments [5].

Studies utilizing molecular-based methods for identification have noted the presence of toxigenic *B. cereus* in various West African-based condiments such as *iru* [18], *ugba* [19], and *mbuja* [20]. However, there are few studies on the microbiological quality and safety of *okpeye*, particularly at the retail level. There is also limited information on the antibiotic resistance profiles of bacteria isolated from indigenous African fermented foods. The aim of this study was, therefore, to investigate the microbiological safety of commercial *okpeye* samples available for retail sale in southeast Nigeria.

2. Methods

2.1. Sample collection

A total of eighteen *okpeye* samples were purchased from six different markets in two cities, Owerri (Relief, World Bank, Ekeukwu) and Anambra (Ose, Bridgehead, Ochanga) in southeast Nigeria. Three samples were obtained from different sellers and composited to represent one sample per market. Samples were collected between December 2021 and January 2022.

2.2. Microbiological analysis

Tenfold dilutions were prepared in Maximum Recovery Diluent (MRD; Oxoid, UK). Appropriate dilutions were plated out in duplicate on Plate count agar (PCA; Oxoid) incubated at 27 °C for 24 h to enumerate the population of aerobic mesophiles, Mannitol salt phenol-red agar (MSA; Millipore, UK) incubated at 37 °C for 24–48 h for the enumeration of *Staphylococcus* and MacConkey agar (MAC; Oxoid) plates incubated at 37 °C for 24–48 h to enumerate *Enterobacteriaceae*.

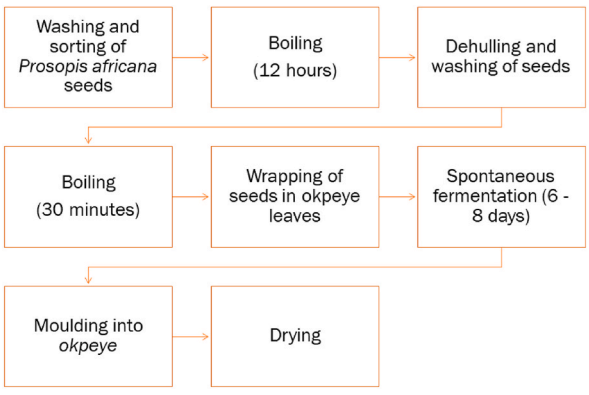

Fig. 1. Local production technology for the fermentation of *Prosopis africana* seeds into *okpeye*.

2.3. Identification of the predominant bacteria from okpeye

To identify predominant microorganisms associated with *okpeye* fermentation, colonies selected from the highest dilution on (PCA, MSA and MAC) plates were purified by streaking on tryptone soya agar (TSA, Oxoid) plates several times. Bacterial isolates were first identified and selected based on phenotypic methods (Gram stain, catalase, and oxidase tests). A total of 59 isolates were selected, and the chromosomal DNA of each isolate was extracted using InstaGene™ Matrix (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. An almost complete portion (*ca.* 1400 bp) of the 16S *rRNA* gene was amplified using primers fd1 (10 μM) and 1492R (10 μM) as described by Ref. [21] and reaction mixture and conditions as described by Ref. [9]. For some isolates, *gyrB* and *rpoB* genes were amplified and sequenced to discriminate between closely related *Bacillus* species where this could not be achieved with the full-length 16S *rRNA* gene sequences. Amplification reactions of the *gyrB* and *rpoB* genes were conducted as described by Refs. [22,23] respectively. Positive PCR amplicons were confirmed by agarose gel electrophoresis (1.5 % w/v) and purified using the QIAquick PCR Purification kit (Qiagen, Crawley, UK). The purified products were sequenced using either the forward or reverse amplification primers (3.2 μM). Identification of isolates at the genus or species level was carried out by performing a search in the EzBiocloud database for the 16S *rRNA* sequences [24].

The BLAST tool (National Centre for Biotechnology, MD, USA) was used to analyse the *gyrB* and *rpoB* sequences in the GenBank sequence database.

2.4. Cytotoxin, enterotoxin and emetic toxin production

2.4.1. Detection of haemolysin activity

Twenty-eight (28) isolates were screened for haemolytic activity on blood agar containing 5 % (w/v) sheep blood (Oxoid) by streaking a loopful of 24 h cultures. Blood agar plates were incubated at 35 °C for 24 h and checked for haemolysis surrounding bacterial growth [25].

2.4.2. Enterotoxin, cytotoxin, and emetic toxin production by B. cereus

The amplification of genes encoding all toxin genes was carried out in a 25 μL reaction mixture comprising 2 μL of chromosomal DNA, 12.5 μL of SuperFi Master mix (Invitrogen, UK), 2.5 μl of forward and reverse primers (10 μM), and 5.5 μL of sterile molecular grade water. The PCR amplification included initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing temperature as applicable to the primer set (Table 1), and extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. Positive PCR product amplicons were confirmed using agarose gel electrophoresis. *Bacillus cereus* (B13) was used as a control for PCR amplifications except for the emetic toxin gene *ces*B [26].

Table 1
Primers used in this study.

Target[a]	Primer	Sequence (5' – 3')	Annealing temperature	Amplicon size (bp)	Reference
16S RNA	fd1	AGAGTTTGATCCTGCCTCAG	55	1463	[27]
	1492R	CGGTTACCTTGTTACGACTT			[28]
gyrB	GyrB-F	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	66	900	[22]
	GyrB-R	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT			
gyrB	Up1-F	GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA	66	920	[29]
	Up2- R	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT			
rpoB	rpoB-F	AGGTCAACTAGTTCAGTATGGAC	51	560	[30]
	rpoB-R	AAGAACCGTAACCGGCAACTT			
nheA	NheA-F	TACGCTAAGGAGGGGCA	56	480	[31]
	NheA-R	GTTTTTATTGCTTCATCGGCT			
nheB	NheB-F	CTATCAGCACTTATGGCAG	54	754	
	NheB-R	ACTCCTAGCGGTGTTCC			
nheC	NheC-F	CGGTAGTGATTGCTGGG	54	564	
	NheC-R	CAGCATTCGTACTTGCCAA			
hblA	HBLA1	GTGCAGATGTTGATGCCGAT	56	301	[32]
	HBLA2	ATGCCACTGCGTGGACATAT			
hblC	L2A	AATGGTCATCGGAACTCTAT		731	
	L2B	CTCGCTGTTCTGCTGTTAAT			
hblD	L1A	AATCAAGAGCTGTCACGAAT		411	
	L1B	CACCAATTGACCATGCTAAT			
cytK	CK-F-1859	ACAGATATCGG (GT)CAAAATGC	54	809	[33]
	CK-R-2668	TCCAACCCAGTT (AT) (GC)CAGTTC			
entFM	EntFM-F	ATGAAAAAAGTAATTTGCAGG	60	1269	[34]
	EntFM-R	TTAGTATGCTTTTGTGTAACC			
cesB	Ces-F	GGTGACACATTATCATATAAGGTG	58	1271	[35]
	Ces-R	GTAAGCGAACCTGTCTGTAACAACA			

2.5. Antibiotic susceptibility testing

The susceptibilities of twenty-eight bacterial isolates from *okpeye* to a panel of commonly used antibiotics were determined using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid) using the guidelines described by Ref. [36]. All antibiotic discs used were obtained from Oxoid and included: ampicillin (10 μg), penicillin (10 μg), tetracycline (30 μg), gentamicin (10 μg), streptomycin (25 μg), erythromycin (15 μg), ciprofloxacin (5 μg), vancomycin (30 μg), chloramphenicol (30 μg) and kanamycin (30 μg). The breakpoints of the Clinical and Laboratory Standards Institute standards [37] were used to classify zones of inhibition as susceptible or resistant. Resistant and intermediate-resistant isolates were considered non-susceptible as previously described [38]. An isolate was described as multidrug-resistant when it was non-susceptible to at least three different classes of antibiotics.

2.6. Statistical analysis

Microbial counts obtained in the collected samples were converted to base-10 logarithms. The chi-square test was used to determine differences in the distribution of the isolates according to location. All statistical analyses were performed using Microsoft Excel version 16 [39].

3. Results

3.1. Microbial counts in commercial okpeye samples

Commercial samples of *okpeye* collected from six different markets in Anambra and Imo states respectively showed similar patterns in microbial counts with a few exceptions. (Fig. 2). Aerobic mesophile counts on PCA ranged between 10^5 to 10^6 CFU/g in samples from all markets. Presumptive staphylococci were only present in *okpeye* samples collected in the three markets in Imo State (OWB, OEM, ORM). In these samples, numbers ranged from 10^5 to 10^6 CFU/g (Fig. 2). Presumptive Gram-negative bacteria isolated on MacConkey agar were detected in all *okpeye* samples at levels ranging from 10^5 to 10^6 CFU/g.

3.2. Microbial identification

The 16S *rRNA* genes of the predominant isolates were sequenced and based on sequence similarity to reference strains in the EzBiocloud database could be identified to genus and species level in most cases (Table 2). Of the 59 isolates, two predominant genera were identified. The first was *Bacillus* (47.4 %). At species level, isolates were identified as *B. amyloliquefaciens/siamensis* (10.1 %), *B. cereus/paramycoides* (16.9 %), *B. tequilensis/cabrialesii* (8.47 %), *B. atrophaeus/velezensis* (5.08 %), *B. lichenformis/haynesii* (3.39 %),

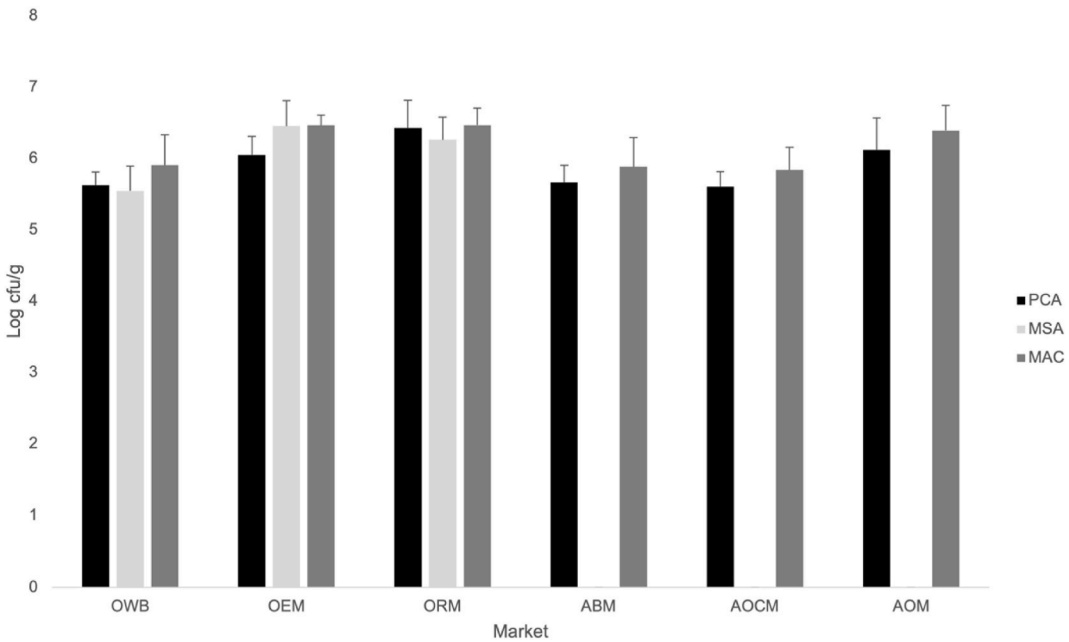

Fig. 2. Bacterial counts in *okpeye* samples obtained from local markets in Imo (OWB; Owerri World Bank Market, OEM; Owerri Ekeukwu Market, ORM; Owerri Relief Market) and Anambra (ABM; Anambra Bridgehead Market, AOCM; Anambra Ochanga Market, AOM; Anambra Ose Market) states in the southeast of Nigeria. Aerobic mesophile counts were determined on Plate Count Agar (PCA), *Staphylococcus* counts on Mannitol Salt agar (MSA) and *Enterobacteriaceae* counts on MacConkey agar (MAC).

Table 2
Identification of microorganisms from *okpeye* using 16s rRNA sequencing and *gyrB/rpoB* sequencing for closely related species.

Location[a]	Isolate code	Identification 16S rRNA gene sequencing	Identification *gyrB/rpoB* sequencing
OWB	OW3	*Bacillus tequilensis*	
OWB	OW4	*Heyndrickxia oleronia*	
OWB	OW5	*Shigella flexneri*	
OWB	OW6	*B. cabrialesii/tequilensis*	*B. tequilensis*
OWB	OW7	*B. cabrialesii/tequilensis*	
OWB	OW8	*B. atrophaeus/velezensis*	
OWB	OW9	*B. atrophaeus/velezensis*	*B. velezensis*
OWB	OW10	*Escherichia fergusonni*	
OWB	OW11	*B. licheniformis*	
OWB	OW12	*Oceanobacillus caeni*	
OWB	OW13	*O. caeni*	
OWB	OW14	*B. tequilensis/cabrialesii*	
OWB	OW15	*B. amyloliquefaciens/siamensis*	*B.amyloliquifaciens*
OWB	OW16	*Paenibacillus barcinonensis*	
OWB	OW17	*Lynsinibacillus xylanilyticus*	
OWB	OW18	*Staphylococcus simulans*	
OWB	OW19	*B. cereus/paramycoides/paranthracis*	*B. cereus*
OEM	OW20	*B. cereus/paramycoides/paranthracis*	*B. cereus*
OEM	OW21	*B. cereus/paramycoides/paranthracis*	*B. cereus*
OEM	OW22	*B. cereus/paramycoides/paranthracis*	*B. cereus*
OEM	OW23	*B. tequilensis/cabrialesii/maquosorum*	
OEM	OW24	*B. licheniformis/haynesii*	*B. licheniformis*
ORM	OW25	*B. velezensis*	
ORM	OW26	*S. nepalensis*	
ORM	OW27	*B. amyloliquefaciens*	
ORM	OW28	*S. nepalensis*	
ORM	OW29	*B. amyloliquefaciens*	
ORM	OW30	*S. nepalensis*	
ORM	OW31	*B. siamensis*	
ORM	OW32	*S. cohnii/ureilyticus*	*S. cohnii*
ORM	OW33	*S. nepalensis*	
ORM	OW34	*B. cereus/paramycoides*	*B. cereus*
ORM	OW35	*B. gaemokensis/anthracis*	*B. anthracis*
ORM	OW36	*S. nepalensis*	
Location	Isolate code	Identification 16S rRNA gene sequencing	Identification *gyrB/rpoB* sequencing
AOCM	A1	*B. cereus/paramycoides*	*B. cereus*
AOCM	A2	*Lysinibacillus xylanilyticus*	
AOCM	A3	*B. cereus*	
AOCM	A4	*Vagacoccus lutrae*	
AOCM	A5	*S. cohnii*	
AOCM	A6	*S.nepalensis*	
AOCM	A7	*S. simulans*	
AOCM	A8	*S. simulans*	
AOCM	A9	*S. simulans*	
AOM	A10	*S. simulans*	
AOM	A11	*B. cereus/paramycoides*	*B. cereus*
AOM	A12	*V. carniphilus*	
AOM	A13	*S. cohnii*	
AOM	A14	*S. simulans*	
AOM	A15	*B. amyloliquefaciens*	
AOM	A16	*B. amyloliquefaciens*	
ABM	A17	*B. cereus/toyonensis*	*B. cereus*
ABM	A18	*S. simulans*	
ABM	A19	*S. simulans*	
ABM	A20	*S. cohnii*	
ABM	A21	*B. cereus/paramycoides*	*B. cereus*
ABM	A22	*S. simulans*	
ABM	A23	*S. simulans*	
ABM	A24	*S. simulans*	
ABM	A25	*B. amyloliquefaciens*	

a Sample collection sites in Owerri (OWB; Owerri World Bank Market, OEM; Owerri Ekeukwu Market, ORM; Owerri Relief Market) and Anambra (ABM; Anambra Bridgehead market, AOCM; Anambra Ochanga Market, AOM; Anambra Ose market).

[a] Sample collection sites in Owerri (OWB; Owerri World Bank Market, OEM; Owerri Ekeukwu Market, ORM; Owerri Relief Market) and Anambra (ABM; Anambra Bridgehead market, AOCM; Anambra Ochanga Market, AOM; Anambra Ose Market).

B. anthracis (1.69 %) and *B. siamensis* (1.69 %). The second predominant group (42.3 %) comprising *Staphylococcus* species were identified as follows; *S. simulans* (16.94 %), *S. nepalensis* (10.17 %) and *S. ureilyticus/cohnii* (6.78 %). Other bacterial species identified included *Oceanobacillus caeni* (3.39 %), *Paenibacillus barcinonensis*, *Vagacoccus lutrae* (3.39 %), *Lynsinibacillus xylanilyticus* (3.39 %), *Shigella flexneri* (1.69 %) and *Escherichia fergusonni* (1.69 %). While most of the isolates could be identified based on their 16S *rRNA* sequences, the results also revealed organisms with close percentage similarities. For example, *B. atrophaeus/velezensis*, *B. lichenformis/haynesii*, *B. amyloliquefaciens/siamensis*, *B. cereus/paramycoides and S. ureilyticus/cohnii*. Further identification was carried out by sequencing of *gyrB* and *rpoB* genes of the above isolates and they were identified as *B. velezensis*, *B. lichenformis*, *B. amyloliquefaciens*, *B. cereus and S. cohnii* (Table 2). The microbial profiles of the isolates from *okpeye* samples varied according to the market and city. There was an association between the distribution of bacterial genera and both the city and market where samples were collected ($p < 0.001$, χ^2 test). *Bacillus* was the dominant genera in Owerri samples while *Staphylococcus* species were dominant in samples from Anambra. For example, *B. lichenformis*, *B. tequilensis*, *and B. velezensis* were specific to samples collected in Owerri and none were isolated from any of the markets in Anambra. *Staphylococcus simulans* were more predominant in the Anambra Ochanja and Bridgehead markets compared to the Anambra Ose market but none was found in any Owerri market. However, *S. nepalensis* was present only in Owerri Relief and Anambra Ochanja markets. However, there were also similarities across markets in both cities. For example, *B. cereus* and *B. amyloliquefaciens* were isolated from all the samples in Owerri and Anambra markets (Table 2).

3.3. Distribution of toxin genes among B. cereus from okpeye

All *B. cereus* isolates were screened for the detection of toxin-producing genes. Five out of the ten (10) *B. cereus* tested possessed all genes encoding the production of non-haemolytic enterotoxin complex (*nheA*, *nheB*, *nheC*) (Table 3). However, all *B. cereus* tested positive for the presence of *nheA*, while *nheB* and *nheC* were amplified in only seven of these isolates. Seven *B. cereus* were positive for the presence of all three genes encoding haemolysin BL complex (*hblA*, *hblC*, *hblD*). Similarly, 70 % of the *B. cereus* isolates possessed the diarrhoeal toxins *cytK* and *entFM* although this did not always correspond with the presence of genes encoding the other enterotoxin genes (Table 3). The emetic toxin (*cesB*) was detected in three *B. cereus* isolates. Only one *B. cereus* (O21), isolated from the *okpeye* samples purchased from the Ekeukwu market in Owerri (OEM), tested positive for all toxin genes screened (Table 3). However, three isolates (O22, O34, A11) possessed all toxin genes screened except for the emetic toxin (*Ces*). The toxin genes *nheA, nheB, hblA, hblC, hblD*, *cytK* and *entFM* were detected in all *B. cereus* isolated from the Owerri samples.

3.4. Antibiotic resistance and haemolytic activity of isolates from okpeye

Twenty-eight isolates were tested for antimicrobial susceptibility with ten antibiotics: including ampicillin, penicillin, tetracycline, gentamicin, streptomycin, ciprofloxacin, erythromycin, chloramphenicol, kanamycin, and vancomycin. Irrespective of the locality of the samples, most of the isolates were susceptible to the antibiotics tested (Table 4). Interestingly, all tested microorganisms were resistant to ciprofloxacin except for two isolates from the Anambra market (A2 and A7). The microorganisms isolated from the Owerri market samples were susceptible to most of the antibiotics tested compared to isolates from *okpeye* samples purchased from Anambra. *Bacillus tequilensis* (O6, O7, O23) was susceptible to almost all antibiotics tested except for tetracycline and ciprofloxacin. Also, *B. amyloliquefaciens (*O27), showed multiple drug resistance and was only susceptible to tetracycline, gentamicin, and streptomycin while (A16) identified from the Anambra market was susceptible to all antibiotics except ciprofloxacin. *Staphylococcus cohnii (*A13*)* and *V. carniphilus (*A12*)* from the Anambra market were also resistant to most of the antibiotics tested (Table 4).

From the twenty-eight isolates tested, twenty-three (82.1 %) showed complete haemolytic activity on blood agar. Two isolates (O22 and A22) showed partial haemolysis while three (O11, O27 and A12) were the only ones that showed no haemolysis (Table 4).

Table 3
PCR analysis of toxin genes from *Bacillus cereus* isolated from *okpeye*.

Isolate code	Microorganisms	*nheA*	*nheB*	*nheC*	*hblA*	*hblC*	*hblD*	*entFM*	*cytK*	*cesB*
O19	*B. cereus*	+	+	–	+	+	+	+	+	+
O20	B. cereus	+	+	–	+	+	+	+	+	+
O21	*B. cereus*	+	+	+	+	+	+	+	+	+
O22	*B. cereus*	+	+	+	+	+	+	+	+	–
O34	*B. cereus*	+	+	+	+	+	+	+	+	–
A1	*B. cereus*	+	–	+	–	–	–	–	–	–
A3	*B. cereus*	+	–	–	–	–	–	–	+	–
A11	*B. cereus*	+	+	+	+	+	+	+	+	–
A17	*B. cereus*	+	–	+	+	+	+	–	–	–
A21	*B. cereus*	+	+	+	–	–	–	+	–	–
B13[b]	Control	+	+	+	+	+	+	+	+	–

+: positive, -: negative [b:] positive control [26].
Enterotoxin genes (*nheA, nheB, nheC, hblA, hblC, hblD, cytK, entFM*) and emetic toxin (*cesB*).

Table 4
Haemolysis and antibiotic susceptibility of some microorganisms isolated from *okpeye*.

Isolate Code	Isolate	Haemolysis[a]	Antibiotic resistance profile[b]									
			Am	P	T	G	S	Ci	E	Ch	K	V
O6	*B. tequilensis*	+	S	S	S	S	S	R	S	S	S	S
O7	*B. tequilensis*	+	S	S	S	S	S	R	S	S	S	S
O8	*B. velezensis*	+	S	S	S	S	S	R	S	S	S	S
O9	*B. velezensis*	+	S	S	R	S	S	R	S	S	S	S
O11	*B. licheniformis*	–	R	S	S	S	S	R	S	R	S	S
O11	*B. licheniformis*	–	R	S	S	S	S	R	S	R	S	S
O14	*B. tequilensis*	+	R	R	S	S	S	R	S	I	R	R
O15	*B. amyloliquefaciens*	+	S	S	S	S	S	R	S	S	S	S
O22	*B. cereus*	–	R	R	S	S	S	R	S	S	S	R
O23	*B. tequilensis*	+	S	S	S	S	S	R	S	S	S	S
O24	*B. lichenformis*	+	R	R	S	S	S	R	S	R	R	S
O27	*B. amyloliquefaciens*	–	R	R	S	S	S	R	R	R	R	R
O31	*B siamensis*	+	S	S	R	S	S	R	S	S	S	S
O35	*B.anthracis*	+	R	R	S	S	S	R	R	S	S	R
A11	*B. cereus*	+	R	R	R	S	S	R	S	S	S	R
A16	*B. amyloliquefaciens*	+	S	S	S	S	S	R	S	S	S	S
O26	*S. nepalensis*	+	S	S	S	S	S	R	R	S	I	R
O32	*S. cohnii*	+	S	S	S	S	R	R	S	S	S	R
A7	*S. simulans*	+	S	S	S	S	R	S	S	S	S	I
A13	*S. cohnii*	+	I	R	S	S	R	R	R	S	R	R
A22	*S. simulans*	–	R	R	S	S	S	R	S	S	S	I
O4	*H. oleronia*	+	R	R	S	S	S	R	S	R	R	R
O12	*O. caeni*	+	S	S	S	S	S	R	S	S	S	S
O13	*O. caeni*	+	S	S	S	S	S	R	S	S	S	S
O17	*L. xylanilyticus*	+	S	S	S	S	S	R	R	S	S	S
A2	*L. xylanilyticus*	+	S	S	S	S	R	S	S	S	R	I
A4	*V. lutrae*	+	I	R	R	S	S	R	I	S	R	R
A12	*V. carniphilus*	–	R	R	R	S	R	R	R	R	R	R

[a] (+) = Haemolysis, (−) = No haemolysis
[b] Am= Ampicillin, P= Penicillin G, T= Tetracycline, G= Gentamicin, S= Streptomycin, Ci= Ciproflaxin, E= Erythromycin, Ch= Chloramphenicol, K= Kanamycin, V= Vancomycin. S= susceptible, I intermediate, R resistant. The results were translated and zones of diameter were compared with both [36] and [37] standard zone diameter breakpoints.

4. Discussion

The microbiological quality and safety of *okpeye* available for retail sale were investigated. In this study, bacterial counts were relatively high (6–7 $\log_{10}$ CFU/g) for aerobic mesophiles in samples obtained from all markets. These results agree with several reports where total aerobic counts up to 10 $\log_{10}$ CFU/g have been observed in indigenous fermented condiments such as *ugba*, *daddawa*, and *maari* [12,19,40]. The presumptive staphylococci count in *okpeye* also ranged from 6 to 7 $\log_{10}$ CFU/g in all samples collected. This corroborates the report by (5) where staphylococci count ranged between 6 and 13 $\log_{10}$ CFU/g in *bikalga*, *soumbala* and *ntoba mbodi*. High aerobic counts in food products at the retail level typically indicate the neglect of good hygiene and sanitary measures during production, and handling [41]. However, environmental factors such as the increase in pH during the fermentation of protein-rich raw materials for condiment production will also influence the microbial population. The increase in pH during fermentation will select for microbial species that can tolerate alkaline conditions [5]. Thus, accurate identification of the microbial population present must be considered while evaluating quality and safety.

Okpeye, like other indigenous African fermented food condiments, is dominated by *Bacillus* species. *Bacillus* was the predominant genus in commercial samples collected from all six markets. The dominance of *Bacillus* spp. in these foods has been attributed to their ability to degrade plant proteins into peptides and amino acids [42,43]. These amino acids can be utilised as carbon and nitrogen sources releasing ammonia or ammonium hydroxide, resulting in high pH values and characteristic odour associated with fermented condiment [4,44,45]. In addition, the long cooking times used to soften the seeds before fermentation will select for spore formers such as *Bacillus* [43].

Previous reports have highlighted *B. subtilis* as the most predominant *Bacillus* species in *okpeye* [46,47]. This contrasts with this study where *B. amyloliquefaciens* and *B. cereus* were the most prevalent. However, these studies relied on phenotyping alone for identification. Interestingly [48], also observed the dominance of *B. subtilis* in *okpeye* using 16S *rRNA* identification. Although *B. subtilis* was not identified in this study, it should be noted that some Bacillus species exhibit very high levels of 16S *rRNA* gene sequence similarities. In particular, *Bacillus subtilis* has been reported to have a 16S *rRNA* gene sequence similarity of >98.7 % to species such as *B. amyloliquefaciens*, *B. tequilensis,* and *B. velenzensis* identified in this study [49].

The microbial profile associated with the commercial *okpeye* samples will be influenced by bacterial sources and processing conditions along the value chain. Bacterial species associated with the raw material, ingredients, processing utensils, environment and the processors themselves will vary by locality [13,50]. The differences observed in this study may be attributed to these variations and

highlight the need for further studies that characterise bacterial species along the processing chain.

The predominance of *B. cereus* in retailed *okpeye* is similar to observations in *ugba* reported by Ref. [19]. There are other reports of the presence of *B. cereus* in indigenous fermented condiments available for retail sale [14,51]. This suggests that *B. cereus* is frequently associated with these foods. It has been stated that the presence of *B. cereus* in most traditional fermented condiments could be a potential source of food poisoning and therefore a public health concern [52]. However, it is also possible that potentially pathogenic bacteria such as *B. cereus* may contribute to the fermentation process. When investigating the microbial diversity of *ugba*, the predominant species identified was *B. cereus* [19]. Similarly, two *B. cereus* strains isolated from *daddawa* were found to possess probiotic attributes [53].

In this study, *B. cereus* was identified based on sequencing of 16S *rRNA* and the *rpoB* or *gyrB* genes. This approach has been reported to be more discriminatory in the identification of *B. cereus* [54,55]. The presence of toxin-producing genes in some of these isolates serves to validate these results. However, it should be noted that the identification of *B. cereus sensus stricto* is complicated due to its genetic proximity to seven other closely related species in the *B. cereus* group [56,57]. Therefore, whole genome sequencing (WGS) is a more reliable tool for the identification and confirmation of *B. cereus sensu stricto* [58,59].

All ten *B. cereus* isolates in this study had at least one gene or gene complex associated with the production of an enterotoxin or an emetic toxin. In particular, genes encoding the non-haemolytic (Nhe) toxin complex (*nheA*, *nheB*, *nheC*) appear to be quite widespread in *B.* cereus from indigenous fermented condiments. In our study, 50 % of isolates possessed the complete Nhe complex. In an earlier report by Ref. [48], the gene encoding Nhe was detected in all *B. cereus* isolates from *okpeye*. Similarly [26], reported that all *B. cereus* isolates from *soumbala* and *bikalga* had all three *nhe* genes. Nhe is a major enterotoxin and is present in almost all pathogenic *B. cereus* isolates [60]. [33] reported that *B. cereus* isolates from vegetables harboured and expressed a high percentage of genes encoding non-haemolytic enterotoxins. In this study, genes encoding the enterotoxins CytK and Enterotoxin FM were also detected in *B. cereus*. While potential cereulide producers have previously been isolated from African fermented food condiments [54], to the best of our knowledge, this study is the first to report this observation in *okpeye*. Emetic toxin-producing genes have previously been detected at different low rates (1.5–17.2 %) in *B. cereus* strains isolated from various food sources [61,62] and the different prevalence rates could be attributed to the differences in the properties in the food [63].

Bacillus spp. has historically been reported in the literature to be responsible for the development of the organoleptic and biopreservative characteristics associated with fermented condiments. However, with the use of molecular techniques for identification, a high occurrence of other groups of bacteria has been reported in these foods [5,19]. *Staphylococcus* was the second most predominant genus after *Bacillus* in samples obtained from all markets. *Staphylococcus* spp. identified in this investigation included *S. nepalensis, S. cohnii* and *S. simulans*. This observation is similar to the report of [5] who frequently isolated staphylococci in alkaline fermented condiments collected from Burkina Faso and Central Africa. Also [51], noted the presence of staphylococci in retail-level *ogiri*. Unlike (5), no *S. aureus* was identified in this study.

Although the possible role and contribution of these identified *Staphylococcus* species during the fermentation of *okpeye* were not determined, their presence is of concern as their presence in food could be indicative of poor hygiene practices from the producers as well as methods of display in the market. Isolation of *Staphylococcus* spp. from commercial okpeye suggests possible contamination from leaf wrappings, fermentation materials, and/or personal hygiene of the handlers [64]. Given the frequency of their recovery from alkaline fermented foods [5], postulated that staphylococci could be considered secondary microorganisms during fermentation. However, a clear indication of their role in fermentation has not been established. Nevertheless, it can be suggested that the presence of *Staphylococcus* species may contribute to some biochemical activities such as those related to the degradation of proteins and lipids that some species can perform during meat fermentation to produce sausages [65,66]. Other non-*Bacillus* and *Staphylococcus* species identified in this study included *Escherichia fergusonni* and *Vagacoccus lutrae* which are considered to be of public health importance.

Some secondary microorganisms including pathogenic bacteria and their toxins like *B. cereus*, *Staphylococcus* spp., *E. coli, Salmonella* spp., *Shigella* spp., and *Clostridium* spp. have been identified in West African fermented seed condiments giving rise to food safety and public health concerns [4,14]. In addition to toxin production from the *B. cereus* isolates, haemolytic activity is an important determinant in assessing the virulence potential of bacteria. Most of the isolates tested displayed haemolytic activity on blood agar. The presence of potentially pathogenic bacteria, and virulence factors provides further evidence that fermented condiments such as *okpeye* may be considered vehicles for the transmission of microbial hazards.

Effective antibiotic therapy is the main treatment for bacterial infections; therefore, the rise of antibiotic-resistant infections is a major threat to public health [67]. Food remains an important route for the transmission of bacteria to consumers. In indigenous fermented foods, bacteria are often consumed in large quantities [15]. Thus, consideration of the antibiotic susceptibility of these bacteria has become an important aspect of food safety. However, there is a paucity of data on the antibiotic resistance profiles of bacteria from African indigenous fermented foods [13].

Consistent with previous studies, most bacteria were susceptible to gentamicin, erythromycin, kanamycin, and chloramphenicol [10,68,69]. The phenotypic susceptibility observed suggests that these isolates do not harbour antibiotic-resistance genes which can be transferred to other bacteria. However, widespread resistance to fluoroquinolones and glycopeptides which are noted as critically important antibiotics by the World Health Organisation was observed [70]. In this study, 58 % of the isolates tested were resistant to vancomycin. This is of concern, as vancomycin had long been designated a 'last resort' antibiotic for several Gram-positive and multi-drug resistant infections [71]. Furthermore, multidrug resistance was observed in some isolates including *B. cereus*. The indiscriminate use of antibiotics in the medical, veterinary, and agricultural sectors contributes to selective pressure for antibiotic-resistant bacteria in the environment. These results support urgent calls on the need for large-scale surveillance studies to investigate the prevalence of antibiotic-resistant bacteria in indigenous fermented foods.

5. Conclusion

Okpeye, like other indigenous fermented condiments in Africa, is an important contributor to food security. The results from this study provide data on the predominant bacterial species associated with commercial *okpeye* available for retail sale in southeastern Nigeria. The high prevalence of *B. cereus* and *Staphylococcus* spp. observed in these foods stress the importance of creating awareness and educating local producers of this condiment about personal and environmental hygiene. Currently, local production of *okpeye* still relies on spontaneous fermentation. In addition, poor control of processing conditions, inadequate hygienic practices and post-processing handling are major drawbacks associated with the safety of fermented condiments [15]. The absence of microbiological standards and limitations in monitoring and surveillance of indigenous fermented foods in Nigeria compromise their quality and safety and risk consumers' health. The production technology can be improved by the use of stable starter cultures with well-characterised technological attributes [13]. As *okpeye* is a source of technologically important bacteria, further studies should focus on the identification and characterisation of potential starters that can be used to improve fermentation technology. There is also a need for extension programmes to train local processors on how to use these starters appropriately for controlled fermentations and in good manufacturing practices.

Data availability

The nucleotide sequences of the isolates have been deposited in GenBank. GenBank accession numbers assigned to the isolates in this study are OR975920 (OW3), OR976498 (OW4), OR978592 (OW5), OR979681 (OW7), OR97682 (OW8), OR97683 (OW10), OR97684 (OW11), OR980946 (OW14), OR981820 (OW13), OR981821 (OW16), OR981822 (OW12), OR981876 (OW17), OR981877 (OW20), OR982365 (OW18), OR981878 (OW19),

OR981883 (OW23), OR982103 (OW25), OR982321 (OW26), OR982406 (OW27), OR982460 (OW29), OR982461 (OW31), OR982603 (A1), OR982648 (A2), OR982670 (A3), OR982675 (A4), OR982676 (A5), OR982686 (A7), OR982687 (A8), OR982688 (A9), OR982689 (A10), OR982693 (A11), OR982694 (A12), OR982695 (A13), OR982702 (A14), OR983142 (A17), OR983143 (A16), OR983144 (A15), OR983145 (A15), OR983251 (A22), OR983255 (A24), OR983256 (A25), OR984134 (OW28), OR984135 (OW30), OR984136 (OW33), OR984137 (OW36), OR984138 (A6), OR984139 (A18), OR984140 (A19), OR984141 (A23).

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CRediT authorship contribution statement

Ijeoma M. Agunwah: Writing – original draft, Investigation, Funding acquisition, Conceptualization. **Chika C. Ogueke:** Writing – review & editing. **Justina N. Nwosu:** Writing – review & editing. **Amarachukwu Anyogu:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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