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Original Research Article

Molecular screening of potential *Bacillus* species bacteriocin starter cultures isolated from Nigerian fermented condiments

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Abstract

Traditional fermented food condiments in Nigeria are majorly produced by using protein-rich leguminous seeds via spontaneous fermentation or back slopping. However, health risks such as the production of biogenic amines, presence of toxigenic genes and food borne pathogens remains major concerns of public health significance. Hence, the need for the production of safe condiments using non-pathogenic food-grade starter cultures. Isolation and characterization of organisms from spontaneously fermented condiments made from *Parkia biglobosa, Ricinus communis, Pentaclethra macrophylla* and *Prosopis africana* was done using standard methods. Phenotypic and biochemical characterizations was down using established protocols while molecular identification was done using 16S rRNA sequencing. Results obtained showed that the microbial load (cfu/ml) recorded from the spontaneously fermented condiments ranged from: 2.1×10^{7}-5.2×10^{12}, 15.2×10^{7}-4.4×10^{10}, 4.4×10^{10}-4.4×10^{12} and 6.4×10^{7}-1.6×10^{12} for *P. biglobosa, R. communis, P. macrophylla* and *P. africana* respectively. Identification of isolates revealed *Bacillus*. However, 22.8% of the *Bacillus* isolates harboured HblA and HblD genes while 40.9 % showed bands for the presence of HblC genes. In addition, the percentage occurrence of 18.1%, 27.2% and 50% was obtained for the presence of NheA, NheB and NheC genes respectively while none of the *Bacillus* isolates showed bands for the presence of Cytk and EM1 genes. More so, five *Bacillus* isolates were identified to be non-toxigenic, showing bands only for Bac, Osbo and Spas genes which are bacteriocin producing genes. Put together, the identified non-toxigenic food-grade *Bacillus* species recorded in this study can be recommended as potential bacteriocin starter cultures for the fermentation of microbiologically safe food products.

Keywords: *Condiments, Bacillus subtilis, fermentation, polymerase chain reaction, P. biglobosa*

INTRODUCTION

The genus *Bacillus* are characterized as a heterogeneous collection of phenotypically large

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collection of spores forming, aerobic, rod-shaped, Gram-positive bacteria that have undergone reclassification in recent times following tangible advances in molecular biology (Rai *et al*., 2016; Celandroni *et al*., 2019; Ogwuanyi and Okpara, 2019). Literatures have documented the used of *Bacillus* species in food, medical, pharmaceutical and industries due to their wide range of physiological attributes and their ability to produce different types of bioactive compounds such as bacteriocins (e.g., iturins, nisins, subtilins, surfactins, etc), enzymes (e.g., lichenase, amylases, xylanases, etc) and antibiotics (e.g., bacitracin, surfactin, pumilin, bacilysin, etc) (Kaskoniene *et al*., 2017; Dimidi *et al*., 2019 and Gopikrishna *et al*., 2021). Different species of *Bacillus* have also been reported to produce nutraceuticals and carotenoids that can be used for the synthesis of a large variety of human and animal health supplements (Mohammed *et al*., 2014; Tanaka *et al*., 2014 and Takano *et al.*, 2016). However, their identification by conventional methods has often proven difficult due to the close similarities among the closely related species that share almost the same morphological, biochemical and sugar fermentation characteristics, hence, the need for molecular identification (Almasoud *et al*., 2014; Shu and Yang, 2017).

The microbiota in the alkaline fermentation process of condiments is a function of the hygienic status of the production environment, the utensils, the raw materials used as well as and the handlers as fermentation processes is often spontaneous (Sabate *et al*., 2013). The reports of Olasupo and Okorie. (2019) reported that a heterogenous population of microbiota, including the technologically important ones as well as pathogenic organisms liable to synthesize toxic by-products such as mycotoxins, emetic toxins, haemolytic toxins, ethyl carbamate and biogenic amines into the spontaneously fermented condiments will have negative implications for the public and safety of the fermented products (Adedeji *et al*., 2017; Adekoya *et al*., 2019 and Owusu-Kwarteng *et al*., 2020). More so, the microbiota of the fermented condiments is of great significance to the end- product characteristics and qualities.

Bacteriocins are naturally ribosomally synthesized antimicrobial peptides (AMPs) by the genus *Bacillus* and examples includes: nisin, subtilin, gengycin, enteriocin, pediocin and surfactin (Rai *et al*., 2016; da Silva *et al*., 2018; Kimura and Yokoyama, 2019). Due to their ability to be used as effective biopreservatives, there is a vast growing interest in the search for bacteriocin producing *Bacillus* species that can be harnessed for biopreservative purposes in food and biotechnology industries (Dabire *et al*., 2018; Dabire *et al.*, 2020 and Kim *et al*., 2023). In addition, food grade *Bacillus* sp. also have a wide antimicrobial activity against food spoilage and food-borne pathogens (Nath *et al.*, 2015; Sharmila and Vidya, 2015; Caulier *et al*., 2019). According to the reports of Delesa (2017) and Flynn *et al.* (2019) chemical food preservatives can also inhibit the growth of food spoilage and pathogenic food-borne organisms, but they usually alter the organoleptic, nutritional and mineral qualities of the preserved foods which can have adverse health effect on humans when consumed. Therefore, this study was designed to investigate potentially bacteriocin producing *Bacillus* sp. starter cultures from spontaneously fermented Nigeria condiments by screening them for the absence of toxigenic genes and haemolysis since only food grade *Bacillus* sp. can be used for food fermentation, their safe status is of significant necessity.

MATERIALS AND METHODS

Isolation and purification of bacterial strains

One (1)g of each condiment sample was suspended in 9ml of sterile distilled water and homogenized using a vortex mixer. The samples were further diluted in a 12-fold serial dilution

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and 0.1ml of appropriate dilutions of 10^5 and 10^7 were plated on sterile nutrient agar plates using the spread plate method and incubated aerobically at 37 ^oC for 24 hr. Representative dominant colonies were transferred into sterile tryptone soy broth and incubated aerobically at 37 ^oC for 24 hr. A loopful from the broth cultures were aseptically streaked on tryptone soy agar and also incubated aerobically at 37 ^oC for 24 hr to obtain pure cultures. The pure isolates were kept on nutrient agar slants and stored at -20 ^oC for further analyses.

Phenotypic characterization

Pure bacterial cultures of 18-24 hr old were Gram stained and examined for cell motility and morphology such as: shape, elevation, margin and colour. Biochemical characterizations such as: oxidase, catalase, spore formation and sugar fermentation were also carried out using the method described by Pershakova *et al.* (2018) with respect to Bergey's manual of Determinative Bacteriology.

Haemolytic activity on blood agar

The bacterial isolates were streaked on blood agar plates for the presence or absence of haemolysis. The cultured plates were incubated at 37 ^oC for 24-48 hr.

Molecular characterization

Genomic DNA extraction

The genomic DNA of the representative bacterial isolate were extracted using the Quick-DNA ^{TM} miniprep plus kit (catalog Nos: D4068 and D4069) from Zymo Research by aseptically scooping 24 hr old bacterial cultures into 1.5mL Eppendorf tubes containing bashing beads to which 200µl sterile nuclease free water has been added. The genomic DNA was extracted in accordance to the manufacturer's instructions and stored at -20 ^oC for further analyses.

Polymerase chain reaction (PCR) analysis

Table 1: Primers used in this study

Bacteria group	Primers	No of nucleotides	Primer sequence (5'-3' orientation)	Expected band size (bp)	References
Bacillus spp.	B-K1/F3	19	TCACCAAGGCRACGATGCG	1114+	Dabire *et al*., 2021
	B-K1/R5	18	CGTATTCACCGCGGCATG	1114+	Dabire *et al*., 2021
B. subtilis group	B sub 5F B sub 3R	19 22	AAGTCGAGCGGACAGATGG CCAGTTTCCAATGACCCTCCCC	595+ 595+	Dabire *et al*., 2021
	HblA: F	20	AAGCAATGGAATACAATGGG	1154	Ahaotu *et al*., 2013
Haemolytic enterotoxin A, B and D	HblA: R	22	AGAATCTAAATCATGCCACTGC	1154	Ahaotu *et al*., 2013
	HblC: F	24	GATAC(T,C)AATGTGGCAACTGC	740	Ahaotu *et al*., 2013
	HblC: R	24	GATAC(T,C)AATGTGGCAACTGC	740	Ahaotu *et al*., 2013
	HblD: F	20	ACCGGTAACACTATTCATGC	829	Ahaotu *et al*., 2013
	HblD: R	20	GAGTCCATATGCTTAGATGC	829	Ahaotu *et al*., 2013
Non-haemolytic enterotoxin A, B and C	NheA:F	20	GTTAGGATCACAATCACCGC	755	Ahaotu *et al*., 2013
	NheA:R	20	ACGAATGTAATTTGAGTCGC	755	Ahaotu *et al*., 2013

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	NheB:F	21	TTTAGTAGTGGATCTGTACGC	743	Ahaotu *et al*., 2013
	NheB:R	20	TTAATGTTCGTTAATCCTGC	743	Ahaotu *et al*., 2013
	NheC:F	19	TGGATTCCAAGATGTAACG	683	Ahaotu *et al*., 2013
Cytotoxin K	CytK: F	24	ACAGATATCGG(G,T)CAAAATGC	809	Ahaotu *et al*., 2013
	CytK: R	21	TCCAACCCAGTTWSCAGTTCD	809	Ahaotu *et al*., 2013
Emetic toxin	EM1: F	31	GACAAGAGAAATTTCTACGAGCAAGTACAAT	unknown	Ahaotu *et al*., 2013
	EM1: R	32	GCAGCCTTCCAATTACTCCCTTCTGCCACAGT	unknown	Ahaotu *et al*., 2013
	Bac F	21	AAGAGTTTGATCCTGGCTCAG	1000-1500+	Dabire *et al*., 2021
Bacteriocins	Bac R	20	CTACGGCTACCTTGTTACGA	1000-1500+	Dabire *et al*., 2021
	Osbo F	23	CCTCATGACCAGGACTTCGCCTT	1200+	Dabire *et al*., 2021
Subtilosins	Osbo R	20	CGGTGCCGAGCGCTTCAGGT	1200+	Dabire *et al*., 2021
	SpaS F	28	CAAAGTTCGATGATTTCGATTTGGATGT	152+	Dabire *et al*., 2012
subtilins	SpaS R	27	GCAGTTACAAGTTAGTGTTTGAAGGAA	152+	Dabire *et al*., 2021

Key: F= forward primer; R= reverse primer

The 16S rRNA used for *Bacillus* identification, specific primers used for the detection of toxigenic and bacteriocin genes are documented in Table 1.

Table 2: Polymerase chain reaction (PCR) protocol for the detection of 16S rRNA, toxigenic and bacteriocin producing genes

Primers	Initial denaturation	30/35 cycles	Annealing	Extension	Final extension
B-K1 F/R	94 °C for 5min	94 °C for 1m	55 °C for 45s	72 °C for 45s	72 °C for 10m
B-Sub F/R	94 °C for 5 min	94 °C for 1min	60 °C for 45s	72 °C for 45s	72 °C for 10 min
HblA F/R	94 °C for 2 min	94 °C for 1min	55 °C for 1 min	72 °C for 2 min	72 °C for 5 min
HblC F/R	94 °C for 2 min	94 °C for 1min	55 °C for 1 min	72 °C for 2 min	72 °C for 5 min
Hbl D F/R	94 °C for 2 min	94 °C for 1min	58 °C for 1 min	72 °C for 2 min	72 °C for 5 min
NheA F/R	94 °C for 1 min	94 °C for 1min	55 °C for 1 min	72 °C for 2 min	72 °C for 5 min
Nhe B F/R	94 °C for 2 min	94 °C for 1min	56 °C for 1 min	72 °C for 2 min	72 °C for 5 min
Nhe C F/R	94 °C for 2 min	94 °C for 1min	55 °C for 1 min	72 °C for 2 min	72 °C for 5 min
CytK F/R	94 °C for 2 min	94 °C for 1min	55 °C for 1 min	72 °C for 2 min	72 °C for 5 min
EM1 F/R	95 °C for 15 min	95 °C for 30s	60 °C for 30s	72°C for 1 min	72 °C for 5 min
Bac F/R	95 °C for 5 min	94 °C for 1min	55 °C for 30 s	72 °C for 45 s	72 °C for 10 min
Osbo F/R	94 °C for 3 min	94 °C for 30s	50 °C for 30s	65 °C for 1 min	72 °C for 10 min
spaS F/R	95 °C for 3 min	94 °C for 30s	55 °C for 30 s	65 °C for 1 min	72 °C for 10 min

Key: F= forward primer; R= reverse primer

The polymerase chain reaction (PCR) protocols for Table 1 above is presented in Table 2 (Ahaotu *et al*., 2013 and Dabire *et al*., 2021). All the genes were amplified in a Thermocycler under the conditions stated in Table 2 and amplified products cooled at 4 °C. Sterile nuclease free water was used as a control.

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Identification of isolates at species level

The isolates were differentiated to species level using the method of Ahaotu *et al.* (2013) and Dabire *et al.* (2021) by amplifying the 16S rRNA using the *Bacillus* specific 16S primers (B-K1/F5 and B-K1/R3) (Table 1) under the conditions presented in Table 2 above using an Eppendorf master cycler (Vapo product). The PCR mixture (25µl) consisted of: One Taq mix (12.5µl), B-K1 forward primer (1.25µl), B-K1 reverse primer (1.25µl), template (5 µl) and H_2O (5 µl). All amplified products (amplicons) were cooled at 4 °C

Gel electrophoresis

In order to separate the DNA fragments of each amplicons, 10µl of each amplicon was mixed with 2 µl of purple loading dye and pipette into a 2.0% agarose gel containing 1×TBE buffer. Quick load purple 100 bp DNA ladder (molecular makers) were used as standards. The electrophoresis (BIORAD) was run under 400A, 90 V for 35 min. Distinctive bands were viewed inside a SyngeneG-box (model: Chemi XX9, rating: 240V, 50Hz) and stored appropriately on the computer system.

Sanger sequencing of bacteriocin producing *Bacillus* sp.

Non-toxigenic bacteriocin producing *Bacillus* sp. were sequenced using the Sanger sequencing technique. Ninety-six (96) well plates of cycle sequencing were used for the Sanger sequencing; the amplicons were purified using ethanol/EDTA precipitation method. Twenty-five (25) ng of amplicons were used to perform the cycle sequencing using the Applied Biosystem (ABI) 3500 according to the manufacturer's instructions. The evolutionary history was inferred using the unweighted pair group method with arithmetic mean (UPGMA method) (Villeger *et al*., 2017).

RESULTS AND DISCUSSION

Phenotypic identification

This study provides information on non-toxigenic bacteriocin producing *Bacillus* sp. that can be used as starter cultures in food fermentation processes.

There are documented literatures on the isolation and characterization of *Bacillus* sp. from spontaneously fermented food condiments as observed in this study (Compaore *et al*., 2013; Fernandez *et al*., 2013; Kang *et al*., 2014; Loranjo *et al*., 2019 and Batiha *et al*., 2021).

Highest and lowest bacterial count was recorded in *ogiri* (15.2×10^7 cfu/ml) and *okpehe* (1.2×10^{12} cfu/ml) respectively.

The dominance of *B. subtilis* in alkaline fermented condiment samples either from Africa or Asia origin have been well documented by several as obtained in this study (Kang *et al*., 2014; Sharmila and Vidya, 2015; Fira *et al*., 2018; Nwagu *et al*., 2020; Dabire *et al*., 2021 and Kim *et al*., 2023). All the analysed bacterial isolates in this study were Gram positive, motile, spore formers, catalase positive and oxidase negative organisms. Some of the plates showed distinctive colonies of one, two or three organisms while some swarmed over the plates, displaying their motility characteristics. The colours of the isolates were either white, cream or butter colour with raised or flat elevation.

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Table 3: Microbial count and morphological characteristics of isolated bacterial sp.

S/N	Isolate Code	Colony count	No of distinct colonies	Shape	Margin	Elevation	Colour
1	IWOI2.10ia	4.8×10^{10}	1	Irregular	Filamented	Raised	Cream
			2	Irregular	Undulate	Raised	Cream
2	IWAO2.12ii	2.4×10^{12}	1	Round	Entire	Raised	Cream
3	IWAO1.12ii	1.2×10^{12}	1	Round	Entire	Flat	Cream
4	IWAO1.7i	6.0×10^{7}	1	Punctiform	Entire	Raised	Cream
			2	Curved	Entire	Convex	Cream
5	OOI1.7i	15.2×10^{7}				Raised	
			1	Irregular	Entire	Raised	Cream
			2	Punctiform	Entire	Raised	Cream
6	OOI3.7i	2.4×10^{7}	1	Round	Entire	Raised	Cream
			2	Irregular	Undulate	Raised	Cream
7	OKOI4.10ia	2.4×10^{10}	1	Irregular	Undulate	Flat	White
8	IPOI5.10ia	Swarming	Swarming	Irregular	Lobate	Flat	Cream
9	IPOI3.12ia	2.1×10^{7}	1	Round	Entire	Flat	White
10	OKOI7.12ia	1.6×10^{12}	1	Irregular	Undulate	Raised	Light orange
11	IPOI2.7iia	1.2×10^{7}	1	Punctiform	Entire	Raised	Cream
12	OKOI5.12iia	2.4×10^{12}	1	Irregular	Lobate	Raised and slimy	Cream
			2				
13	OKAO4.12i	2.0×10^{12}	1	Irregular	Entire	Raised	Cream
14	OKAO8.12ia	1.2×10^{12}	1	Round	Entire	Raised and slimy	Cream
15	OGOA10.7iia	12.0×10^{7}	1	Irregular	Lobate	Raised	White
16	OGOA8.7ib	6.4×10^{10}	1	Round	Entire	Raised	Cream
17	OGOA1.7iia	4.8×10^{7}	1	Punctiform	Lobate	Raised	Cream
18	OKAO6.12i	4.4×10^{12}	1	Irregular	Filamentous	Raised	Cream
19	IWOI4.7iia	6.8×10^{7}	1	Irregular	Lobate	Raised	Cream
			2	Round	Entire	Raised	cream
20	UAKO4.12ii	4.4×10^{12}	1	Round	Entire	Flat	Cream
			2	Punctiform	Entire	Raised	Cream
			3	Punctiform	Entire	Raised	Butter colour
21	IWOI5.12iia	3.2×10^{12}	1	Round	Entire	Undulate	Cream
22	OOI2.7ib	2.8×10^{7}	1	Swarming	Undulate	Entire	Cream
			2	Punctiform	Entire		

Table 3 documents the microbial count and morphological characteristics of the isolated bacterial sp. from spontaneously fermented condiments.

Table 4: Gram's staining, biochemical and sugar fermentation tests of the isolated bacterial sp.

S/N	Isolate code	Gram's reaction	Catalase	Oxidase test	Endospore	haemolysis	glucose	maltose	lactose	fructose	mannose	*Probable organism*
1	IWOI2.10ia	+	+	-	+	+	F+ G+	F+G-	F+G-	F+G+	F+ G-	*Bacillus cereus*
2	IWAO2.12ii	+	+	-	+	-	F+ G+	F- G-	F+G-	F+G+	F+ G+	*B. licheniformis*
3	IWAO1.12ii	+	+	-	+	+	F+ G+	F+G-	F+G+	F+G-	F+ G-	*B. cereus*
4	IWAO1.7i	+	+	-	+	+	F+ G-	F+G-	F+G-	F+G+	F+ G+	*B. cereus*
5	OOI1.7i	+	+	-	+	+	F+ G-	F+G+	F+ G-	F+G+	F+ G-	*B. cereus*
6	OOI3.7i	+	+	-	+	+	F+ G+	F+G-	F+G-	F+G-	F+ G+	*B. cereus*
7	OKOI4.10ia	+	+	-	+	+	F+ G+	F+G-	F+ G-	F+G+	F+ G-	*B. cereus*
8	IPOI5.10ia	+	+	-	+	-	F+ G+	F+G+	F+G+	F+G+	F+ G+	*B. subtilis*
9	IPOI3.12ia	+	+	-	+	-	F+ G+	F+G+	F+G+	F+G+	F+ G+	*B. subtilis*
10	OKOI7.12ia	+	+	-	+	-	F+ G-	F+G+	F+G+	F G+	F+ G+	*B. pumilus*
11	IPOI2.7iia	+	+	-	+	+	F+ G+	F+G-	F+G+	F+G-	F+ G-	*B. cereus*
12	OKOI5.12iia	+	+	-	+	+	F+ G+	F+G-	F+G-	F+G+	F+ G	*B. cereus*
13	OKAO4.12ia	+	+	-	+	-	F+ G+	F+G+	F+G+	F+G+	F+ G+	*B. subtilis*
14	OKAO8.12ia	+	+	-	+	+	F+ G-	F+G+	F+ G-	F+G+	F+ G-	*B. cereus*
15	OGOA10.7iia	+	+	-	+	+	F+ G+	F+G+	F+G+	F+G+	F+ G+	*B. subtilis*
16	OGOA8.7ib	+	+	-	+	-	F+ G+	F+G+	F+G-	F+G+	F+ G+	*B. licheniformis*
17	OGOA1.7iia	+	+	-	+	-	F+ G-	F+G+	F+G-	F+G+	F+ G+	*B. pumilus*
18	OKAO6.12i	+	+	-	+	+	F+ G+	F+G-	F+G-	F+G+	F+ G-	*B. cereus*
19	IWOI4.7iia	+	+	-	+	+	F+ G-	F+G+	F+G-	F+G+	F+ G-	*B. cereus*
20	UAKO4.12ii	+	+	-	+	+	F+ G-	F+G+	F+G-	F+G+	F+ G-	*B. cereus*
21	IWOI5.12iia	+	+	-	+	+	F+ G-	F+G+	F+G-	F+G+	F+ G-	*B. cereus*
22	OOI2.7ib	-	-	+	-	+	F+ G+	F+G+	F+G+	F+G+	F+ G+	*Unknown*

The Gram staining reaction, biochemical and sugar fermentation tests result of isolated bacteria from spontaneously fermented condiments is documented in Table 4.

Molecular identification of *Bacillus* species, detection of toxigenic and bacteriocin genes

The PCR result of the 16S rRNA molecular characterization is documented in Table 5. 68% of the

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bacterial isolates harboured one or more toxigenic genes and it is indicated with yellow colour. 5% harboured no haemolytic, emetic genes or bacteriocin producing genes and are highlighted in green colour. In addition, 5% of the isolates are doubtful to belong to the *Bacillus* group (coloured in blue). More so, 23% of the bacterial isolates showed to be the most promising microorganism as they did not harbour any toxigenic genes but possessed *Bacillus* sp., bacteriocin (Bac), subtilosin (Osbo) and subtilisin (Spas) genes and are therefore highlighted in red.

From the toxigenic genes screening in this study, it was recorded that 22.8% of the isolates harboured HblA and HblD genes, 40.9 % showed bands for HblC genes while NheA, NheB and NheC genes had the following percentage of occurrence respectively: 18.1%, 27.2% and 50%. None of the isolates showed band for Cytk and EM1 genes. There are documented literatures that the presence of HblA, C and D, NheA, B and C, EM1 and CytK genes are implicated in food-borne infections and intoxications (Amao-Awua *et al*., 2014; Ademola *et al*., 2018 and Dimidi *et al*., 2019). The study of Ouoba *et al.* (2008) detected NheA, B C and CytK genes in *B. cereus* isolated from ugba. Thorsen *et al.* (2011) also detected NheA, B, C, HblA, C, D, and CytK genes in *B. cereus* isolated from afitin, iru and sonru. In addition, Obadina *et al*. (2008), Ahaotu *et al.* (2013) and Adekoya *et al.* (2019) have also previously documented the detection of toxigenic genes produced by *Bacillus* species isolated from fermented condiments.

Table 5: PCR results for 16S rRNA, toxigenic and bacteriocin genes in the isolated *Bacillus* sp.

S/N	Isolate code	B-K1	B-Sub	HblA	HblC	HblD	NheA	NheB	NheC	CytK	EM1	Bac/Osbo /spaS
1	IWOI2.10ia	þ	þ	þ	Þ	Þ	þ	þ	Þ	×	×	Þ
2	IWAO2.12ii	þ	þ	×	Þ	×	×	×	Þ	×	×	Þ
3	IWAO1.12ii	þ	þ	×	Þ	×	×	×	×	×	×	Þ
4	IWAO1.7i	þ	þ	×	×	×	×	×	×	×	×	×
5	OOI1.7i	þ	þ	×	Þ	×	×	×	Þ	×	×	Þ
6	OOI3.7i	þ	þ	×	×	×	×	×	Þ	×	×	×
7	OKOI4.10ia	þ	þ	×	Þ	×	×	×	×	×	×	×
8	IPOI5.10ia	þ	þ	×	×	×	×	×	×	×	×	Þ
9	IPOI3.12ia	þ	þ	×	×	×	×	×	×	×	×	Þ
10	OKOI7.12ia	þ	þ	×	×	×	×	×	×	×	×	Þ
11	IPOI2.7iia	þ	þ	×	Þ	×	×	×	×	×	×	Þ
12	OKOI5.12iia	þ	þ	þ	×	×	×	×	×	×	×	×
13	OKAO4.12i	þ	þ	×	×	×	×	×	×	×	×	Þ
14	OKAO8.12ia	þ	þ	×	×	×	×	×	Þ	×	×	Þ
15	OGOA10.7iia	þ	þ	×	×	×	×	×	×	×	×	Þ
16	OGOA8.7ib	þ	þ	×	×	×	×	þ	Þ	×	×	Þ
17	OGOA1.7iia	þ	þ	×	Þ	×	×	×	Þ	×	×	Þ
18	OKAO6.12i	þ	þ	þ	Þ	Þ	þ	þ	Þ	×	×	Þ
19	IWOI4.7iia	þ	þ	þ	×	Þ	þ	þ	Þ	×	×	Þ
20	UAKO4.12ii	þ	þ	×	Þ	×	×	×	×	×	×	Þ
21	IWOI5.12iia	þ	þ	þ	×	Þ	þ	þ	Þ	×	×	Þ
22	OOI2.7ii	×	×	×	×	þ	×	þ	Þ	×	×	þ

Table 5 presents the PCR results for 16S rRNA, toxigenic and bacteriocin genes in the isolated *Bacillus* sp.

Key:
B-K1= *Bacillus* group
Bsub= *B. subtilis* group
Hbl A, C and D= Haemolytic enterotoxin A, C and D
NheA, B and C= non-haemolytic enterotoxin A, B and C
CytK= Cytotoxin C
EM1= Emetic toxin
Bac/Osbo/spaS= Bacteriocins/subtilosins/subtilins

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Colour codes and meaning:

Yellow: isolates contain genes for the production of one form of toxin or the other, hence they are unsuitable for further analysis

Blue: Doubtful that the organism is *Bacillus*, hence it was discarded since the focus is on *Bacillus* organisms

Green: isolate does not possess haemolytic genes, however, it neither contain bacteriocin (lacks personal protection)

Red: promising isolates that possess bacteriocin genes, no haemolytic genes, no emetic genes and no cytotoxin genes, hence they were selected for Sanger sequencing

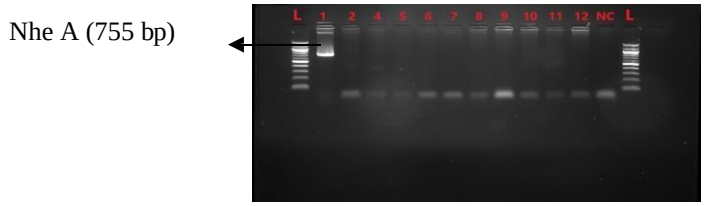

Plate 1: Agarose gel image of isolated *Bacillus* Nhe A genes showing band at 755 bp

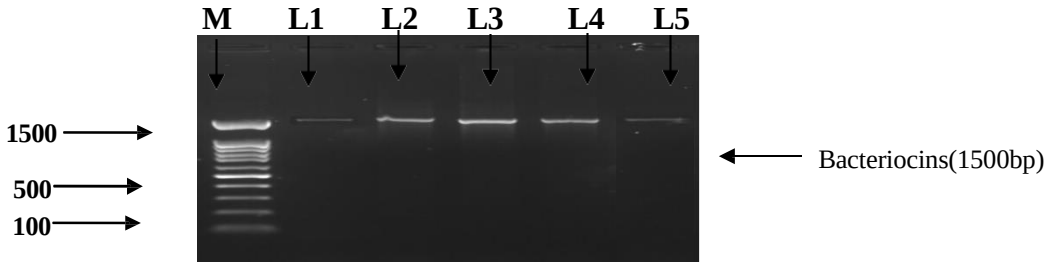

Plate 2: Agarose gel image of identified *Bacillus* bacteriocins (Bac) genes showing bands at 1500 bp

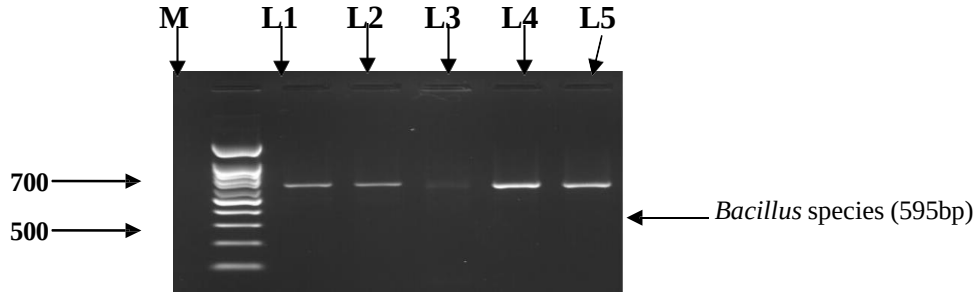

Plate 3: Agarose gel image of *B subtilis* genes (*B. subtilis* group) showing bands at 595 bp

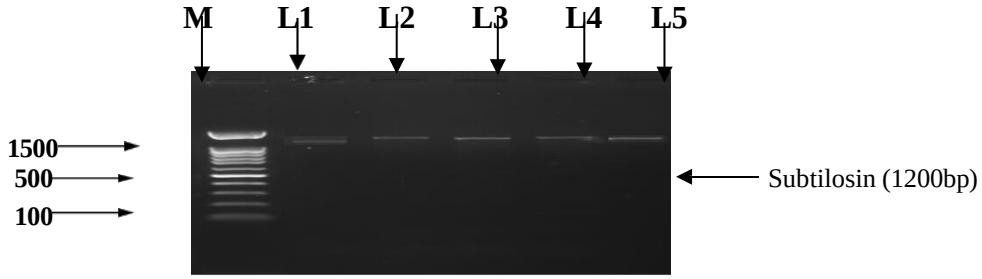

Plate 4: Agarose gel image of *Bacillus* subtilosin genes (Osbo) showing bands at 1200 bp

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Plate 1 above shows the presence of NheA gene in the isolated toxigenic *Bacillus* sp., showing a band size of 755bp while plates 2-4 shows the presence of bacteriocin (Bac), *B. subtilis* and subtilosin (Osbo) genes in the non-toxigenic sequenced *Bacillus*.

The studies of Mishra *et al.* (2017) and Olasupo *et al.* (2019) also documented the detection of toxigenic genes in West African traditional alkaline fermented seed condiments. Therefore, only the isolates that did not harbour any toxigenic genes but only bacteriocin genes was sequenced. The 16S rRNA gene of the isolates were also amplified to differentiate them using the 16S rRNA *Bacillus* specific primers-K1 F/5 and B-K1 R/3 under the conditions given in Tables 2 and 3. This method had earlier been used by Ouoba *et al*. (2008), Ahaotu *et al*. (2013) and Dabire *et al.* (2021). The presence of bacteriocin producing genes in food-grade *Bacillus* species as recorded in this study confirms the claims of O'Connor (2015), Delesa (2017), Kaskoniene *et al*. (2017), Meade *et al*. (2020), Wang *et al*. (2021) and Epparti *et al*. (2022) that the total genomic DNA of *Bacillus* species is allotted to the production of antimicrobial compounds (AMCs). This observation is in line with the result obtained in this study in which five potential bacteriocin *Bacillus* was documented. The studies of Youcef-Ali *et al*. (2014) and Dabire *et al*. (2021) have earlier documented the presence of nisin, pediocin, enterocin, subtilosin and subtilisin bacteriocin genes in lactic acid bacteria and *B. subtilis* isolated from fermented meat (*nham*) and fish mixed with rice (*som-fak*).

Sanger sequencing of potential *Bacillus* bacteriocin starter cultures

The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. This analysis involved 5 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 734 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

The *Bacillus* nucleotide sequences was submitted to the National Centre for Biotechnology Information (NCBI) and the following accession numbers was assigned to them (Table 6) while the phylogenetic tree of the sequenced *Bacillus* is presented in Plate 5.

Table 6: Accession numbers of the sequenced *Bacillus*

S/N	Isolated food grade *Bacillus* sp.	Condiment source	NCBI accession numbers
1	*B. subtilis* IPOI5. 10ia	Iru pete	SUB14529720 1C PP930853
2	*B. subtilis* IPOI3. 12ia	Iru pete	SUB14529720 2C PP930854
3	*B. subtilis* OKOI7.12ia	Okpehe	SUB14529720 3C PP930855
4	*B. paralicheniformis* OKAO4. 12i	Okpehe	SUB14529720 4C PP930857
5	*B. subtilis* OGOA10. 7iia	Ogiri	SUB14529720 5C PP930853

The NCBI accession numbers of the isolated non-toxigenic food grade *Bacillus* sp. from spontaneously fermented condiments is documented in Table 6.

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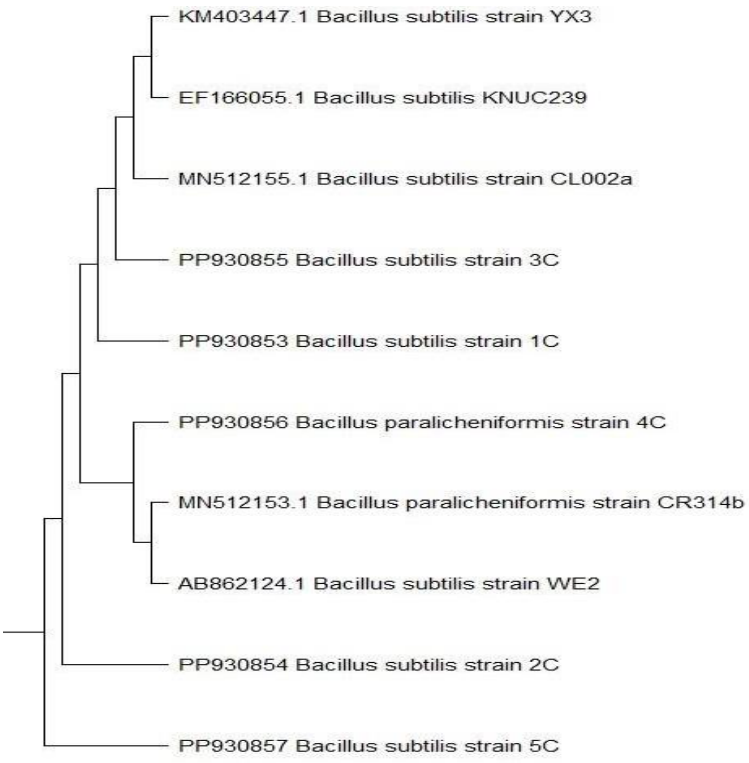

Plate 5: Phylogenetic tree showing the evolutionary relationship of 5 potential bacteriocin *Bacillus* isolated from spontaneously fermented condiments

In addition, literatures have reported the identification of nisin, pediocin, reuterin and toyoncin *B.* bacteriocin producing genes in *pumilus*, *B. mycoides*, *B.toyonensis* and *B.velezensis* that can be harnessed as biopreservative in foods such as meat, fish, soy-sauce, condiments, vegetables and dairy products (Butkhot *et al*., 2019., Batiha *et al*., 2021; Maina *et al*., 2021 and Wang *et al*., 2021).

CONCLUSION

In this study *Bacillus* sp. were isolated from spontaneously fermented Nigerian condiments and screened for the presence of toxigenic and bacteriocin producing genes. Five *Bacillus* isolates showed bands only for the presence of bacteriocin genes and not toxigenic genes. Hence, they were sequenced and accession numbers assigned to them. Therefore, it can be concluded that the sequenced *Bacillus* can be employed as potential bacteriocin starter cultures for the fermentation of microbiologically safe food condiments.

RECOMMENDATIONS

Potential bacteriocin producing *Bacillus* sp. were identified in this present work. Therefore, future studies should be channelled towards isolating, purifying, characterizing and applying the bacteriocins for biopreservative purposes.

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Conflict of interest

The authors declare no conflicts of interest.

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