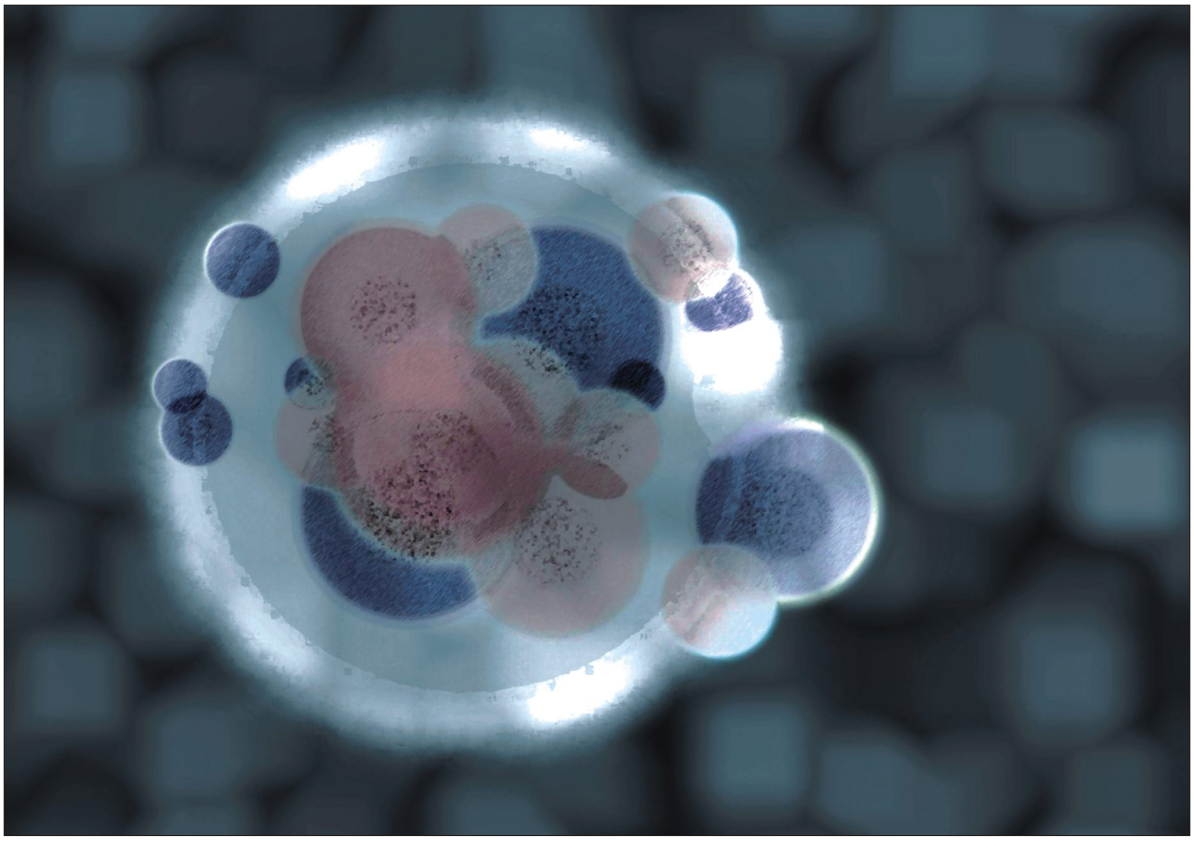

# Journal of Biological Sciences

ISSN 1727-3048

science alert

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OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2022.24.33

# Research Article
# Antimicrobial Activity of Bacteriocin-Like Inhibitory Substances Produced by *Bacillus subtilis* Isolated from "*Iru*"

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## Abstract

**Background and Objective:** '*Iru*' is a traditional condiment obtained by spontaneous alkaline fermentation of African locust bean (*Parkia biglobosa*) seeds. *B. subtilis* is the most predominant microorganism associated with the fermentation of '*Iru*' which antimicrobial substances such as peptides, antibiotics and bacteriocins. This work is aimed at isolation, identification, screening of the antimicrobial activity and partial purification of the Bacteriocin-Like Inhibitory Substances (BLIS) from *B. subtilis* obtained from '*Iru*'. **Materials and Methods:** Twenty-four '*Iru*' samples were collected from different '*Iru*' sellers in different markets in Oyo town. Isolation was done using the Luria-Bertani Agar medium. Identification was carried out using cultural, morphological, biochemical and molecular characterization. Agar well diffusion method was used for the antimicrobial activity. Partial purification of BLIS was done by Ammonium sulphate precipitation. The technological properties and the tolerance to bile salt were also determined. Identification of bioactive compounds was done using Gas Chromatography-Mass Spectrometry (GC-MS) and High-Performance Liquid Chromatography (HPLC). **Results:** A total of 59 bacteria were isolated, 13 were identified to be *Bacillus subtilis*. Five *Bacillus subtilis* (BSAAJ3, BSFAJ3, BSDAJ3, BSFAK2 and BSAAW3) inhibited *Staphylococcus aureus* while *B. subtilis* (BSFAK4) inhibited *E. coli*. *B. subtilis* (BSFAK4) had the highest protease, lipase, esterase, hemolytic potential and tolerance to bile salt. The GC-MS analysis identified 23 bioactive compounds while the HPLC analysis revealed the presence of ribonuclease, cytochrome c, a-chymotrypsinogen and lysozyme as the proteins present. **Conclusion:** These studies revealed that the BLIS obtained from BSFAK4 had the potential to inhibit a wide spectrum of pathogens which will be very useful in the pharmaceutical industry.

Key words: Bacteriocin, antimicrobial activity, bioactive compounds, molecular characterization, bile salt, technological properties

Citation: Olanbiwoninu, A.A., R.O. Fashogbon and K.O. Owoyemi, 2022. Antimicrobial activity of bacteriocin-like inhibitory substances produced by *Bacillus subtilis* isolated from "*Iru*". J. Biol. Sci., 22: 24-33.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Antimicrobial agents are gaining more attention as alternative therapeutics in pharmaceuticals and food industries as preservative[1]. Most of these antimicrobial agents have been discovered from fermented food products such as '*Iru*'. Usually, the seed of the African locust bean (*Parkia biglobosa*) is popularly fermented to a product known as '*Iru*'. *Bacillus* species as the major fermenting microorganisms involved in the fermentation process of most vegetable protein including '*Iru*'. *Bacillus* species are mesophiles with optimal temperatures between 30 and 45°C, while some are thermopiles with optimal temperatures as high as 65°C. Others are psychrophilic. They can grow and sporulate at 100°C and are found to grow in an environment with a pH range between 2 and 11. Mucilage and ammonia are produced by the breakdown of amino acids at the end of the '*iru*' fermentation process by *Bacillus* species[2]. The predominant *Bacillus* sp. involved in the fermentation process of *iru* is mainly *B. subtilis*, *B. megaterium*, *B. firmus*, *B. licheniformis* and *B. circulans*. *B. subtilis* produces a high level of protease, amylase and polyglutamic acid. A significant increase in vitamins, such as thiamine and riboflavin has been observed in '*Iru*' which is due to the riboflavin synthase associated with *Bacillus subtilis*[3]. The production of antimicrobial substances by *Bacillus subtilis* is an essential factor in which they play a crucial role in bacterial interactions including efficient antagonists against pathogens. Bacteriocin is commonly referred to as ribosomally synthesized antimicrobial peptides. They comprise different compounds of proteinaceous substances that are synthesized from every major linage of bacteria[4]. A bioactive compound such as bacteriocins is ribosomally synthesized bactericidal or bacteriostatic peptides produced by certain bacteria. Although Gram-negative bacteria can produce bacteriocins, the vast majority of bacteriocins characterized so far are produced by Gram-positive bacteria[5]. Some bacteriocins can inhibit the growth of similar bacterial species due to their narrow-spectrum activity while some have antimicrobial activity against a wide array of genera. Gram-positive bacteria have a broad spectrum than Gram-negative bacteria[6,7]. As observed with antibiotics, bacteria can develop resistance against bacteriocin principally through modifications of their cell envelope such as alterations in charge and thickness[7].

Due to the rapid rise and spread of antibiotic resistance, there arises the need for continuous surveillance to provide natural but effective alternatives to tackle the menace of antibiotic resistance and reduce its risk. This research is aimed at isolation, identification, screening of the antimicrobial activity and partial purification of the Bacteriocin-Like Inhibitory Substances (BLIS) from *B. subtilis* obtained from '*Iru*'.

## MATERIALS AND METHOD

**Study area:** This study was carried out in Oyo town, which is in the South-Western part of Nigeria. This research project was conducted from June, 2020-January, 2021. The town is situated on latitude 8°00 North of the equator and longitude 4°00 East, having an average daily temperature which ranges between 25°C (77.0°F) and 35°C (95.0°F), covering an area of 28,454 square kilometres and landscape consists of old hard rocks and dome-shaped hills which rise gently from about 500 m in the Southern part and reaching a height of about 1,219 m above sea level in the Northern part.

**Sample collection:** Twenty-four samples of '*Iru*' were purchased from different '*Iru*' sellers at a different market in Oyo town, Oyo State, Nigeria in a sterile bottle and were transported in ice packs to the laboratory for microbiological analysis. Pre-treatment of the '*Iru*' sample was done by placing 10 g in boiling water for 5 min.

**Isolation and identification of microorganisms:** One gram (1 g) of each pre-treated '*Iru*' sample was weighed and mashed in a stomacher bag containing 9 mL of distilled water using a stomacher machine (Seward STOMACHER® 80 Lab System, USA). One millilitre of the dilution from the stomacher bag was pipetted and transferred into a sterile test tube containing 9 mL of 0.1% peptone water. This process was repeated for each of five sets of test tubes until a dilution of $10^{-6}$. About 1 mL from the dilution $10^{-3}$ and $10^{-4}$ were plated in duplicate into Luria-Bertani Agar medium and incubated at 37°C for 24 hrs. Distinct colonies were subcultured to obtain a pure culture. The pure colonies were subjected to Gram staining, spore staining, oxidase, catalase, starch hydrolysis etc[8].

**Antimicrobial activity assay of *Bacillus subtilis* using agar well diffusion:** The antimicrobial activity of the selected thirteen *Bacillus subtilis* was tested by agar well diffusion method. The prepared test suspension of the 24 hrs old four different test organisms, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas fluorescens* and *Klebsiella pneumonia* was spread onto the surface of the Mueller-Hinton agar medium and a well (10 mm in diameter) bore on the surface of the agar and each well was filled with the cell-free supernatant of the potential bacteriocin producers, respectively. The zone manufacturer's name and location

added inhibition was measured after 24 hrs using a Vernier Caliper (YURI, Gujarat, India)[9].

**Technological properties of *Bacillus* sp. isolated from '*Iru*' protease, lipase, esterase and hemolytic activity of *Bacillus subtilis*:** Skim milk agar (skim milk, 10.0, glucose, 1.0, peptone, 5.0, yeast extract, 2.5 and agar, 15.0 (g $L^{-1}$) was weighed into 1 L (1000 mL) of distilled water, vigorously mixed was used to determine protease enzyme activity while Tributyrin agar (10 g tributyrin, 5 g peptone, 3 g yeast extract and 15 g agar in 1 L of distilled water) was used to determine lipolytic activity[10].

Esterase activity was determined using tween 80 hydrolysis. About 10 g tween 80, 5 g NaCl, 0.1 g calcium chloride dehydrate, 10 g peptone and 20 g agar was dissolved in 1 L of distilled water. All media were autoclaved at 121°C for 15 min and a single streak of 24 hrs old pure culture. Hemolytic activity was determined using 24 hrs old pure cultured were streaked on blood agar. All mediums were incubated for 24 hrs at 35°C[10].

**Bile tolerance:** Pure culture of 18-24 hrs old isolates was inoculated into bile salt liquid medium and incubated at 37°C for 24 hrs. The survival rates of the isolates were measured by taking absorbance at 600 nm using a spectrophotometer[11].

**Production of crude metabolites samples:** The bacteria isolates were inoculated into 250 mL Erlenmeyer flasks (SSG, UK) containing 100 mL Nutrient Broth and incubated at room temperature for 48 hrs with intermittent shaking. The broth culture was filtered to separate the cells. After which equal volume of ethyl acetate was added to the filtrate, mixed well for 10 min and kept for 5 min till the two immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using a separating funnel. The extract was concentrated by removing the solvents under room temperature thereby allowing the solvent to evaporate and leaving the crude extracts. The crude extract was dissolved in DMSO and stored at 4°C[12].

**Purification of metabolites:** Partial purification of metabolites was done by Ammonium sulphate precipitation. The crude extract was saturated with 80% Ammonium sulphate (Mallinckrodt Chemical, Inc., Paris, KY, USA) and continuously stirred for 24 hrs 5°C. The precipitate was separated from the filtrate by centrifugation in a cold state at 20,000 rpm for 1 hr at 4°C. The precipitates were resuspended in 25 mL of 0.05 M Phosphate, phosphate buffer (pH 7.0). Dialysis was followed in a tubular cellulose membrane against 2 L of the same buffer for 18 hrs in Repligen Spectra/Por 3 dialysis tubing (3.5 kD MWCO Rolled tubing, USA)[13].

**Antimicrobial activity assay of bacteriocin:** The antimicrobial activity of the potent 6 *Bacillus subtilis* was tested by agar well diffusion method. The crude bacteriocin was tested against four different test organisms, *Escherichia coli, Staphylococcus aureus, Pseudomonas fluorescens* and *Klebsiella pneumonia* was spread onto the surface of the Muller-Hinton agar medium and a well (10 mm in diameter) was bored on the surface of the agar and each well was filled with the cell-free supernatant of the potential bacteriocin producers, respectively. The zone of inhibition was measured after 24 hrs using a Vernier Caliper (YURI, Gujarat, India)[9].

**Identification and phylogenetic analysis:** The most potent isolate which confirmed the presence of bacteriocin as well as having the highest antimicrobial activity was identified by 16S r DNA sequencing. DNA was extracted and amplified by using Polymerase Chain Reaction (GeneAmp 9700 PCR System Thermal cycler, Applied Biosystem Inc., USA) with 16SrDNA specific primers 8F (5'AGAGTTTGATCCTGGCTCAG 3') and 149r (5'CGGTGTGTACAAGACCC 3') using Veriti® 96 well thermal cycler (Model No. 9902). The PCR reactions were performed as denaturation at 95°C for 1 min and the final extension step at 72°C for 10 min. The PCR amplicon was purified and visualized by gel electrophoresis and sequenced. The 16Sr DNA sequence was analyzed using LITE (version 2.01) and the most similar bacterial species were found in the GenBank by BLAST search (http://www.ncbi.nlm.nih.gov/). The neighbour-joining phylogenetic tree was constructed based on a 16Sr DNA sequence using ClustalW[14,15].

**Gas chromatography and mass spectroscopy (GC-MS) analysis of bioactive compounds:** The metabolite treatment 20 mL of the cell-free supernatant was extracted with 5 mL of a mixture of N-Hexane and HPLC grade Methanol. The extraction was done in double folds. Then, the organic phase was pooled together and later cleaned up by column packing with the use of silica gel and anhydrous Sodium sulphate. The analysis was conducted using an Agilent 7890A gas chromatograph (Agilent Technologies, Inc. 2007-2010, USA) equipped and coupled to Agilent 5975C Mass Spectrometer (USA). The cleaned crude extract was injected under the following chromatographic conditions Column thickness: 0.25 µm, Length: 30 m, Internal diameter: 0.32 mm, Carrier gas: Helium, Flow rate: 1 mL $min^{-1}$, Temperature programme: 80°C

to hold for 2 mins at 10°C $min^{-1}$ to 240°C to hold for 6 min, Library software: NIST LIBRARY 2015[16].

## RESULT

**Isolation and characterization of the '*Iru*' samples:** Fifty-nine *Bacillus* species were isolated from the '*Iru*' samples obtained from four different markets. Identification was done based on their colonial, morphological and biochemical characterization. All the isolates appeared dull-white, irregular, dry with opaque colonies and adhere strongly to the surface of the agar. They were all Gram-positive and catalase-positive. Fifty-four of the *Bacillus* species were able to hydrolyze starch while five could not, 55 tested positives to Voges-Proskauer test while four were negative. Twenty two of the *Bacillus* species isolated were able to utilize citrate while seven were not able. Sixteen were able to utilize mannitol as their sole source of carbon. Thirteen out of the *Bacillus* species were able to grow in NaCl at a concentration of 6.5% while nine isolates were not able to grow in NaCl. After considering the results of the colonial, morphological and biochemical test according to Bergey's manual of determinative bacteriology, 13 of the isolates were *B. subtilis* due to their cell diameter which ranged from 0.6-0.8 μm (Table 1).

**Screening for bacteriocin producers:** The thirteen *B. subtilis* screened for the ability to produce metabolites against the test pathogens (*E. coli*, *S. aureus*, *P. fluorescens* and *K. pneumoniae*) is shown in Table 2. Six out of the 13 *Bacillus subtilis* had zones of inhibition ranging from 9-26 mm with BSAKF4 having the largest zone of inhibition (26 mm) while BSAJF3 had the least (9 mm). BSAJD3, BSAJF3, BSAWA3, BSAJA3 and BSAKF2 inhibited two out of the 4 test pathogens while BSFAK4 inhibited three of the test pathogens

Table 1: Morphological and biochemical characteristics of the bacterial isolates

| Isolate codes | Gram stain | Spore forming | Catalase | Starch hydrolysis | Voges proskauer | Citrate test | Mannitol fermentation | Growth at 6.5% NaCl | Cell diameter (μm) | Probable identity |
|---|---|---|---|---|---|---|---|---|---|---|
| AAJ2 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| AAJ3 | + Rod | + | + | + | + | + | + | + | 0.7 | *Bacillus subtilis* |
| CAJ1 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| DAJ3 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| FAJ1 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| FAJ3 | + Rod | + | + | + | + | + | + | + | 0.6 | *Bacillus subtilis* |
| DAK2 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| FAK2 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| FAK4 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| CDM3 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| EDM3 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| AAW3 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |
| BAW2 | + Rod | + | + | + | + | + | + | + | 0.8 | *Bacillus subtilis* |

+: Positive, -: Negative and ND: Not determined

Table 2: Antibacterial activity of potential metabolites producer using agar well diffusion method all values are expressed as Mean±Standard deviation

| Isolates | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | *Staphylococcus aureus* | *Klebsiella pneumonia* | *Escherichia coli* | *Pseudomonas fluorescens* |
| BSAAJ2 | 1.6±0.01 | 2.4±0.05 | 4.3±0.21 | 1.4±0.15 |
| BSAAJ3 | 17.0±0.13 | 2.1±0.25 | 9.0±0.11 | 2.5±0.01 |
| BSDAJ3 | 11.0±0.21 | 2.6±0.12 | 12.9±0.02 | 5.1±0.26 |
| BSFAJ1 | 3.6±0.42 | 4.3±0.62 | 4.6±0.32 | 2.3±0.22 |
| BSFAJ3 | 16.0±1.12 | 9.3±0.14 | 9.5±0.25 | 6.7±0.55 |
| BSCAJ1 | 3.4±0.31 | 1.9±0.65 | 6.1±0.49 | 3.4±0.22 |
| BSDAK2 | 4.3±0.20 | 3.4±0.18 | 4.9±0.35 | 2.9±0.42 |
| BSFAK2 | 10.0±0.43 | 10.3 ±0.9 | 9.8±0.32 | 6.0±0.29 |
| BSEDM3 | 2.6±0.21 | 3.5±0.64 | 4.8±0.61 | 2.9±0.55 |
| BSFAK4 | 19.5±0.43 | 17.2±1.22 | 20.4±0.43 | 16.5±0.25 |
| BSCDM3 | 2.5±0.17 | 3.1±0.24 | 5.2±0.45 | 4.6±0.52 |
| BSBAW2 | 5.0±0.41 | 1.4±0.39 | 2.1±0.40 | 2.7±0.27 |
| BSAAW3 | 10.4±0.42 | 10.1±0.09 | 11.3±0.18 | 12.0±0.41 |

All values are expressed as Mean±Standard deviation, NZ: No zone of inhibition, BS: *B. subtilis*, AJA-AJF: Sample A, B, C, D, E and F, AJ: Ajegunle market, AKA-AKF: Sample A, B, C, D, E and F, AK: Akesan market, DMA-DMF: Sample A, B, C, D, E and F DM: Dan-Zaria market, AWA-AWF: Sample A, B, C, D, E and F, AW: Awe market

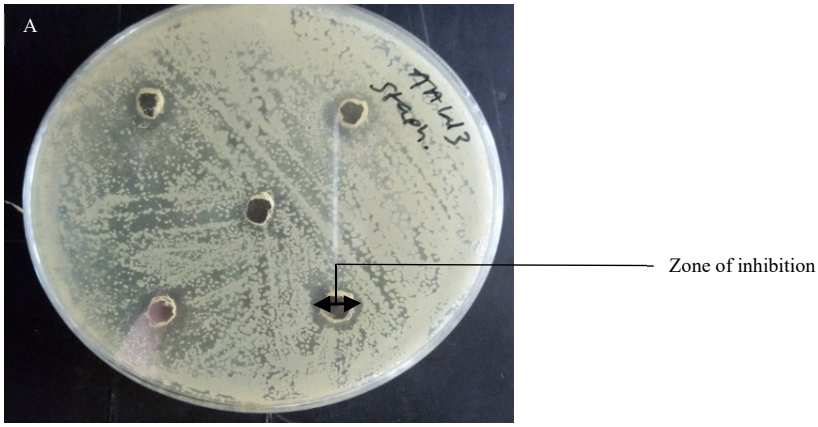


Fig. 1: Zone of inhibition on Mueller-Hinton agar using agar well diffusion method
A-BSAAW3 against *S. aureus*

Table 3: Protease, lipase, esterase and hemolytic activity of *Bacillus subtilis*

| | Zone of inhibition (mm) | | | |
|---|---|---|---|---|
| Isolate codes | Lipase activity | Esterase activity | Protease activity | Hemolytic activity |
| BSAAJ3 | $09^{b}$ | $04^{d}$ | $22^{b}$ | $23^{b}$ |
| BSFAJ3 | $08^{c}$ | $04^{d}$ | $20^{c}$ | $13^{e}$ |
| BSDAJ3 | $08^{c}$ | $06^{c}$ | $15^{e}$ | $11^{f}$ |
| BSFAK2 | $08^{c}$ | $07^{b}$ | $17^{d}$ | $15^{d}$ |
| BSFAK4 | $10^{a}$ | $09^{a}$ | $35^{a}$ | $30^{a}$ |
| BSAAW3 | $04^{d}$ | $04^{d}$ | $14^{f}$ | $21^{c}$ |

Each value is a Mean±Standard error of three replicates. Values in the same column with different letters as superscripts are significantly different by Duncan multiple range tests ($p<0.05$)

(*E. coli*, *K. pneumoniae* and *S. aureus*). However, none of the *B. subtilis* inhibited *Pseudomonas fluorescens.* BSAKF4 has been selected based on the inhibitory effect against most of the indicator strains as well as their zones of inhibition for further studies. Figure 1 shows the zone of inhibition of *Bacillus* sp. against *Staphylococcus aureus*.

**Protease, lipase, esterase and hemolytic activity of *Bacillus subtilis*:** The result showing the protease, lipase, esterase and hemolytic activity of *B. subtilis* is shown in Table 3. The result shows that protease activity ranged from 15-35 mm, lipase activity ranged from 04-10 mm, esterase activity ranged from 04-09 mm while hemolytic activity ranged from 11-30 mm. BSFAK4 had the highest enzyme hemolytic activity while the least was observed in BSDAJ3.

**Resistance to bile salt:** The ability of the *B. subtilis* to survive in different bile concentrations is shown in Fig. 2. The result represents a change of absorbance ($A_{600}$) within 24 hrs. At 0.3% bile concentration the absorbance ranged from 0.791-0.095, BSFAK4 had the highest tolerance while BSAAJ3 had the least. At 0.5%, the absorbance from 0.630-0.057, BSFAK4 had the highest bile tolerance while BSAAJ3 had the least. At 1% the absorbance range from 0.628-0.023, BSFAK4 had the highest bile tolerance while BSAAJ3 had the least. Generally, the highest bile tolerance was observed at 0.3% bile concentration with BSFAK4 having the highest absorbance (0.791). The result represents a change of absorbance ($A_{600}$) within 24 hrs.

**Molecular characterization:** *B. subtilis* FAK4 (BSFAK4) having the highest activity and highest number of bioactive components was selected for molecular characterization as shown in Fig. 3. The amplicons purified and partial sequences of the genes sequenced using the forward and backward primer were identified as *Bacillus subtilis* strain ov2004-03268-01 16S ribosomal RNA gene, the partial sequence showing 99.84% identity with accession number GU585579.1.

**GC-MS analysis of *Bacillus subtilis* FAK4:** In an extract of *Bacillus subtilis* FAK4, 9 compounds were identified of which all belonged to the class fatty acid ester. Among these

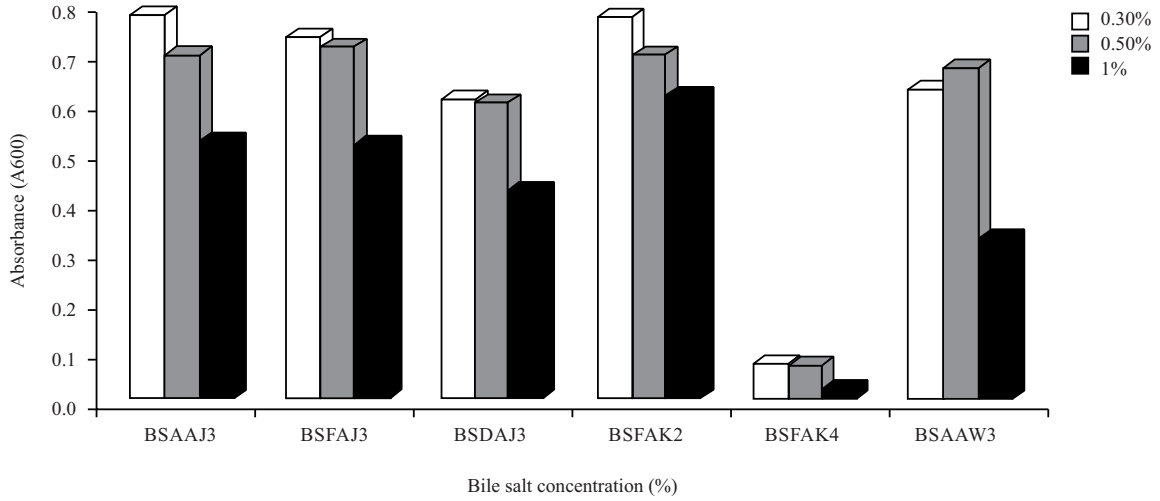


Fig. 2: Survival rate of *B. subtilis* in different bile conditions

Table 4: Bioactive components of *Bacillus subtilis* FAK4

| Pk | RT (min) | Total (%) | Compound names | Molecular formula | Biological activity | Nature of compound |
|---|---|---|---|---|---|---|
| 1 | 6.825 | 1.91 | Decanoic acid, methyl ester | $C_{11}H_{22}O_2$ | Antibacterial and antioxidant | Fatty acid |
| 2 | 9.125 | 45.57 | Dodecanoic acid, methyl ester | $C_{12}H_{24}O_2$ | Antibacterial, antioxidant and antiviral | Fatty acid |
| 3 | 9.625 | 7.14 | Dodecanoic acid | $C_{12}H_{24}O_2$ | Antibacterial, antioxidant and antiviral | Fatty acid |
| 4 | 11.158 | 16.34 | Methyl tetradecanoate | $C_{15}H_{30}O_2$ | Antimicrobial | Fatty acid |
| 5 | 11.534 | 2.33 | Tetradecanoic acid | $C_{14}H_{28}O_2$ | Antioxidant, hypocholesterolemic, cancer preventive and nematicide | Fatty acid |
| 6 | 12.996 | 8.69 | Pentadecanoic acid, 14-methyl-, methyl ester | $C_{17}H_{34}O_2$ | Antimicrobial and antioxidant | Fatty acid |
| 7 | 14.434 | 7.80 | 11-Octadecenoic acid, methyl ester | $C_{19}H_{36}O_2$ | Antibacterial, anti-inflammatory and anti-arthritic | Fatty acid |
| 8 | 14.668 | 1.99 | Methyl stearate | $C_{19}H_{38}O_2$ | Antibacterial and antifoaming agent | Fatty acid |
| | | | Undecanoic acid, 10-methyl-, methyl | $C_{13}H_{26}O_2$ | Antimicrobial | Fatty acid |
| 9 | 15.854 | 2.50 | Methyl 1,2-hydroxy-9-octadecenoate | $C_{19}H_{36}O_3$ | Antibacterial | Fatty acid |
| 10 | 17.220 | 5.73 | Bendazac methyl ester | $C_{16}H_{14}N_2O_3$ | Antimicrobial, antioxidant and anti-inflammatory | Fatty acid |

Pk: Peak area, RT: Retention time

Table 5: HPLC analysis of *Bacillus subtilis* FAK4 bacteriocin

| Peak | Protein | RT (min) | Area | Height | Biological activity |
|---|---|---|---|---|---|
| 1 | Ribonuclease | 48:01:00 | 85330 | 19510 | Nuclease, antitumor, antitumor and anticancer |
| 2 | Cytochrome C | 46:22:00 | 13120 | 2450 | Apoptosis |
| 3 | α-chymotrypsinogen | 44:30:00 | 10760 | 2340 | Proteolysis |
| 4 | Lysozyme | 42:23:00 | 4650 | 920 | Cell lysis |

compounds dodecanoic acid, methyl ester was found to be present as a major constituent with the highest peak area 45.57% and retention time 9.125 min, followed by Methyl tetradecanoate with the peak area of 16.34% and retention time 11.158 min, followed by Pentadecanoic acid, 14-methyl- with the peak area 8.69% and retention time 12.996 min. Compounds such as 11-Octadecenoic acid methyl ester, bendazac methyl ester, methyl 1,2-hydroxy-9-octadecenoate, tetradecanoic acid, methyl stearate and decanoic acid, methyl ester were found to be present at least quantity 8.00% with the peak area 7.80, 5.73, 2.50, 2.33, 1.99 and 1.91% to the retention time 14.434, 17.220, 15.854,11.534, 14.668 and 6.825, respectively (Table 4).

**HPLC analysis of *Bacillus subtilis* FAK4 bacteriocin:** The partially purified bacteriocin of *Bacillus subtilis* FAK4, 4 proteins were identified. Among these proteins, Ribonuclease had the highest peak area with a retention time of 48:01 min followed by Cytochrome C with a retention time of 46:22 min. However, α-chymotrypsinogen and lysozyme with retention time 44:30 and 42:23 min (Table 5).

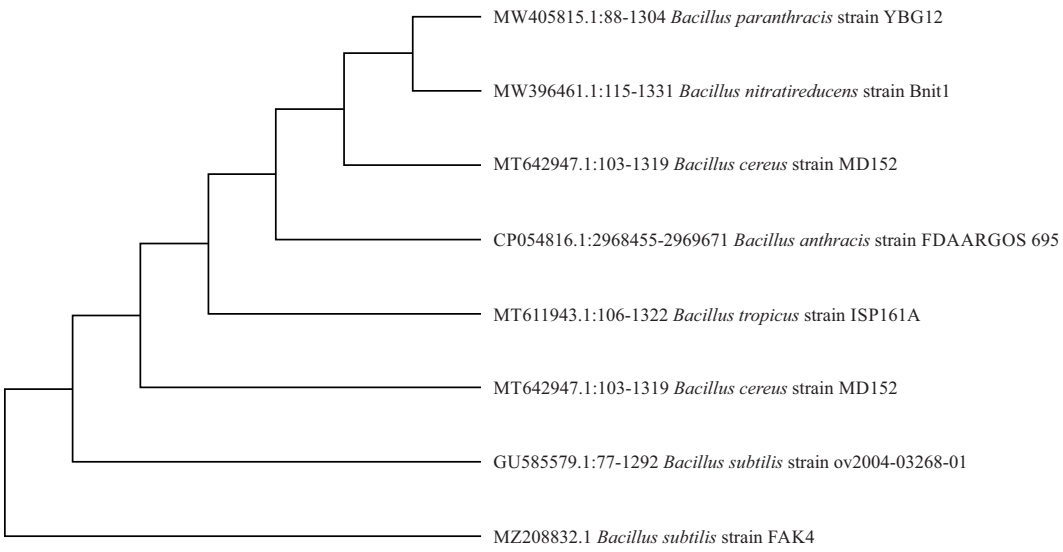


Fig. 3: Phylogenetic tree of *Bacillus subtilis* FAK4

## DISCUSSION

This study is designed to isolate *B. subtilis* the predominant fermenting bacteria in '*Iru*' and screen for its potential to produce bacteriocin. A total of 59 *Bacillus* sp. were obtained from the '*Iru*' samples obtained from 4 different markets. The further test confirms that 13 of the 59 isolates were *Bacillus subtilis* correlates with that of Ozabor and Fadahunsi[8], Powthong and Suntornthitichareon[17] and Ouoba *et al.*[18], who isolated *Bacillus* spp. from '*Iru*' and observed them be Gram-positive, rod-shaped, spore formers and catalase-positive, able to hydrolyze starch, positive to Voges-Proskauer test, utilized citrate, fermented mannitol and were able to grow in NaCl at a concentration of 6.5%. The formation of spores by *Bacillus* sp. occurs when the cell culture reaches the stationary growth phase which occurred as a result of nutritional deprivation or cell density[19]. *Bacillus* species have been attributed to the production of antimicrobial substances such as peptide and lipopeptide antibiotics and bacteriocins[20]. The result obtained in this research supports the findings of Powthong and Suntornthitichareon[17], who reported that *B. subtilis* inhibited the growth of *Staphylococcus aureus*, *Escherichia coli and Klebsiella pneumoniae*. However, these findings contradict the work of Powthong and Suntornthitichareon[17], who reported that *Pseudomonas fluorescens* was inhibited. Metabolites extracted from BSAJD3 showed the lowest zone of inhibition (7.3 mm) against *S. aureus*, while BSAKF4 showed the highest zone of inhibition (17 mm) against *E. coli*. These finding correlates with the result of Al-Ajlani and Hasnain[21] and Ouoba *et al.*[18] where *B. subtilis* inhibited one strain of *S. aureus* and *E. coli*. *B. subtilis* have been reported to be able to produce protein antibiotics such as subtilin, subtilosin and sublancin which are mostly active against Gram-positive bacteria. However, few peptides or protein antibiotics produced by *B. subtilis* are active against Gram-negative bacteria[18,21]. The production of antimicrobial substances and sporulation capacity confer *Bacillus* strains the ability to survive in different habitat[22]. *B. subtilis* is also an important source of enzyme used in food, animal feed and other industrial applications[23]. For *B. subtilis* to effectively function as a probiotic strain, the synthesis of hydrolytic enzymes such as protease is required to break down complex food polymers to generate simpler compounds such as peptides, amino acids, reducing sugars and oligosaccharides which will be further converted through other biological reactions to organic acids and other flavour-impacting and health benefiting compounds[24]. The ability of the *B. subtilis* BSFAK4 to produce protease enzyme could be the reason why they showed antimicrobial activity against the test pathogens. This finding agrees with that of Powthong *et al.*[25] where the enzyme activity of *Bacillus* species was evaluated and all the *B. subtilis* strains were found to be highly proteolytic. The lipase activity revealed that all the *B. subtilis* did not produce lipase enzyme on tributyrin agar while the esterase activity results, revealed that BSFAK4 had high esterase activity. Mashar *et al.*[26] also made similar findings where it was reported that *B. subtilis* had low expression of lipase and esterase enzyme. This is because there is very little amount of suitable plasmids for lipase expression in *B. subtilis*. Thus,

plasmid design and construction studies is a solution to solve the low lipase expression problem in *B. subtilis* as well as research aiming at the optimization of culture conditions to enhance lipase activity[26].

Resistance of the *B. subtilis* to bile salt is a good property because it will be able to pass through the gut which has bile salt with alkaline pH in the intestine[27]. Similar tolerance towards bile was reported by Zaid[28]. It was discovered that *B. subtilis* survived in bile salt concentration from 0.3-2.0% but was unable to survive in 3.0 and 4.0% bile salt. The ability of *B. subtilis* to be resistant to bile salt is caused by the thick peptidoglycan layer and cell wall which are properties of Gram-positive bacteria. This prevents the lysis of Gram-positive bacteria when they are exposed to bile salt[29].

The GC-MS analysis of the Bacteriocin-Like Inhibitory Substance (BLIS) extract of *Bacillus subtilis* FAK4 also showed dodecanoic acid, methyl ester as a major compound present. The *Bacillus subtilis* FAK4 extracts with dodecanoic acid, methyl ester as the most prominent compound, exhibited considerable activity against *Staphylococcus aureus* and *Escherichia coli*. Dodecanoic acid, methyl ester is also known as lauric acid is a saturated medium-chain fatty acid, it is found naturally in various plant and animal fats and oils. It is known to have antimicrobial properties and very low toxicity. This compound was reported with antibacterial, antioxidant and antiviral activity[30]. Aside from the source of isolation, dodecanoic acid, methyl ester was reported with antibacterial activity against *Staphylococcus aureus, Escherichia coli, S. pyogenes, S. mutans* and *K. oxytoca*[31].

The presence of these various bioactive compounds with reported antimicrobial activity against pathogenic organisms justifies the use of metabolites produced by *Bacillus subtilis* against bacterial pathogens. The antimicrobial classification of *B. subtilis* is based on different criteria such as their three-dimensional structure, molecular targets, biosynthesis machinery, biological functions, properties and covalent bonding[32]. However, biosynthetic pathways as previously reported for *Streptococcus* sp. and *Enterococcus* sp. has been used to classify antibacterial molecuels[30].

Based on their synthetic pathway *B. subtilis*, bacteriocin is classified into 5 classes which include Ribosomal Peptides (RPs), Polyketides (PKs), Non-Ribosomal Peptides (NRPs) and volatile compounds. The twenty-three bioactive compounds produced by *Bacillus subtilis* AJA3, *Bacillus subtilis* AJF3 and *Bacillus subtilis* FAK4 are majorly alkanes, sulphur-containing compounds and fatty acids and they all fall under the class of Volatile Organic Compounds (VOCs). Due to the wide variety of volatile organic compounds, this makes them one the most important group of antimicrobial VOCs produced by microbes and they represent about 87 % of the antimicrobial compounds produced by *B. subtilis*[33]. The HPLC analysis revealed the presence of ribonuclease, cytochrome c, a-chymotrypsinogen and lysozyme. The partially purified BLIS had Ribonuclease as the most prominent protein with the highest peak followed by cytochrome c, a-chymotrypsinogen and lysozymes. The identified proteins play various biological and antimicrobial roles. Bacteriocins exhibit antagonistic activities which are synthesized ribosomally[33]. Ribonuclease is involved in many cellular processes including apoptosis and cell defense which was a result of the RNase from the partially purified bacteriocin within *B. subtilis* (termed RNase JI/J2 and RNase Y)[34].

Other *Bacillus* sp. such as *B. amyloliquefaciens* (termed barnase P00648), *B. altitudinis* (balnase, AOAOJ1IDI7), *B. circulans* (P350780) and *B. coagulan* (P37203) were also reported to produce bacteriocin containing RNase[35]. After RNA hydrolysis, the RNase cleave the 3′, the 5′-phosphodiester bond between guanosine 3′-phosphate and the 5′-OH group of the adjacent nucleotide, while generating 2′,3′-cyclic guanosine phosphate thereby inhibiting the reproduction and growth of pathogens[36]. It was discovered that RNase also helps in the detection and characterization of ′*iru*′ lence genes in *S. aureus*[37]. Lysozyme functions as an antimicrobial by enzymatically cleaving a glycosidic linkage of bacterial cell walls peptidoglycan, which leads to cell death[38]. When a Bacteriocin-Like Inhibitory Substance (BLIS) is released by bacteriocin-producing bacteria, they become combined with the corresponding receptor on the surface of the sensitive bacteria to kill the bacteria. The sensitive bactericidal mechanisms of action for bacteriocin include, the pore-forming type also known as pore-forming proteins or Pore-Forming Toxins (PFTs) which are frequently cytotoxic that is they kill cells by producing pores in the membrane of targeted cells. The nuclease type BLIS with DNase and RNase function that has DNase, 16S rRNA and tRNA which it uses to non-specifically digest DNA and RNA of bacteria[39]. The peptidoglycanase type BLIS on the other hand are proteins that digest the peptidoglycan precursor leading to the inability to synthesize peptidoglycan and bacterial death[39]. Comparing the result obtained from these findings with the classification based on the bactericidal mechanism of bacteriocin-like inhibitory substance, it is suggested that the metabolites obtained from BSAKF4 are nuclease type BLIS. This study describes the antimicrobial activity of bacteriocin-like inhibitory substances produced by *Bacillus subtilis* and the technological properties of the *Bacillus subtilis* strains. Bacteriocin-like inhibitory substances produced from *Bacillus subtilis* are natural and nontoxic thereby highly

recommended as an antimicrobial agent. However, there is a need to determine the proteomics of the BLIS from the *Bacillus subtilis* strain. Therefore, Research on the biological activities and proteomics of ribonucleases should be carried out in other to develop a novel drug design, most especially *B. subtilis* RNase should be studied.

## CONCLUSION

The present study shows that the metabolites produced from *B. subtilis* isolated from '*Iru*' have antibacterial activity. *Bacillus subtilis* GU585579.1 showed promising antimicrobial activity and it can be considered as a potential candidate for probiotic organisms.

## SIGNIFICANCE STATEMENT

This study discovered the antimicrobial activity of bacteriocin-like inhibitory substances produced by *Bacillus subtilis* isolated from '*Iru*'. The bacteriocin like substance will be useful in the Pharmaceutical industry. This study will help the researcher to uncover the critical areas of the technological properties and the major bioactive compound present in the Bacteriocin-like substances that many researchers were not able to explore. Thus, a new theory on the biological activities and strain improvement of *Bacillus subtilis* for the optimum production of bacteriocin-like inhibitory substances may be arrived at.

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