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Probiotic Characterization and Antimicrobial Activity of Bacillus Species Isolated from Traditionally Fermented *Parkia biglobosa* Seeds in Nigeria

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Abstract

With the advent of rising antimicrobial resistance and the development of functional foods, efforts have been made to search for indigenous microorganisms with functional and antimicrobial properties. In West Africa, traditionally fermented African locust bean (*Parkia biglobosa*) seeds are an important source of beneficial microorganisms. In this study, the probiotic potential and the antimicrobial activity of the bacterial isolates obtained from the fermented seeds of *P. biglobosa* were evaluated. Culture-dependent methods were used to isolate bacteria, and biochemical methods and 16S rRNA gene sequencing were used to identify each bacterial isolate. Salt tolerance, bile salt tolerance, temperature tolerance, and antimicrobial activity against *Escherichia coli, Proteus vulgaris, Salmonella typhi,* and *Serratia marcescens* were performed to assess the probiotic properties of the microorganisms. Antibiotic susceptibility profiles were also performed. Molecular identification identified eight bacterial isolates of *Klebsiella pneumoniae, Bacillus paramycoides, Bacillus subtilis, Bacillus velezensis* and *Bacillus licheniformis*. Bacillus isolates showed improved probiotic characteristics of growth at 6% NaCl, surviving at 110-112% in bile salts (0.3%) and excellent growth on 25-45°C. However, *K. pneumoniae* isolates had a lower level of bile stress tolerance with 77–78% of strains surviving. Antimicrobial assays results indicated that there were significant difference among isolates ($p<0.001$), in which *B. velezensis* and *B. licheniformis* showed highest antimicrobial property against all test pathogens. The highest inhibition zones were observed against *S. typhi* (20.80 mm) and *E. coli* (20.27 mm). The antibiotic susceptibility profile of these isolates were also good. The results show that fermented *P. biglobosa* is a potential source of functionally important species of Bacillus with probiotic and antimicrobial properties. These indigenous strains can be used as the potential candidates for probiotic foods, biopreservatives development and as supplements for antimicrobial interventions.

Keywords: *Parkia biglobosa*, probiotics, *Bacillus velezensis*, *Bacillus licheniformis*, antimicrobial activity, fermented foods
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1. Introduction

Increased frequency of antimicrobial resistance has heightened the interest in alternative strategies to prevent infections and promote health on a global level. One of these methods, probiotics, has been identified as an important biological agent that not only promotes gastrointestinal health but also regulates immune responses and inhibits pathogenic microorganisms (Mazandarani *et al.,* 2025; Yang *et al.,* 2025). Probiotics are live organisms that have been shown to be beneficial to the host when consumed in sufficient quantities. They exhibit a number of beneficial effects, which include competitive exclusion of pathogens, production of antimicrobial metabolites, immune modulation, strengthening the intestinal barrier and regulating the gut microbial communities (Latif *et al.,* 2023; Amin *et al.,* 2025).

Fermented foods are one of the greatest sources of natural probiotic microorganisms in nature. Traditional fermentation processes are practiced across Africa for centuries and have been used to enhance the nutritional value, digestibility, taste and preservation of foods (Obafemi *et al.,* 2022; Mgbodile & Nwagu, 2023; Adesulu-Dahunsi *et al.,* 2025). Fermentation enhances the production of beneficial microorganisms that can produce bioactive compounds and increase nutrient bioavailability. The indigenous fermented foods are thus important

but underutilized sources of microorganisms which are also probiotics and have adapted to local food systems (Gangakhedkar*et al.,* 2025; Kanimozhi *et al.,* 2026).

Fermented African locust bean (*Parkia biglobosa*) is one of the foods which occupy a prominent position among African fermented foods. In West Africa, this product is referred to by its various names, such as iru, dawadawa or ogiri, and is popular among the people as a traditional condiment (Masters & Kelly, 2024; Golly *et al.,* 2025). There is a complex succession of microorganisms during fermentation, with multiple bacterial groups, especially those belonging to the genus Bacillus species playing a role. These microorganisms play an important role in protein hydrolysis, the development of flavours, reduction of anti-nutritional factors and enhancement of the bioavailability of nutrients (Sharma *et al.,* 2020; Abbaspour, 2024; Sawant *et al.,* 2025).

In recent years, the probiotic properties of spore-forming Bacillus species have come to the fore. Unlike other probiotics, like Lactobacillus and Bifidobacterium, Bacillus species yield very tough endospores that can withstand difficult environmental stress, including bile and gastric acid, heat shock, and dehydration. They are highly resistant, which enhances their shelf life and chances of survival in the stomach (Payne *et al.,* 2024). Thus, Bacillus probiotics have garnered more commercial and scientific interest.

Bacillus species, such as *Bacillus subtilis, Bacillus velezensis,* and *Bacillus licheniformis* demonstrate a high probiotic potential as a source of extracellular enzymes and antimicrobial peptides, bacteriocins, and lipopeptides (Liu *et al.,* 2024; Raman *et al.,* 2025). These metabolites are active against pathogenic bacteria and are good to the gut, maintaining the microbial balance (Luise *et al.,* 2022). Although *P. biglobosa* products, such as fermented locust beans, have been used widely in Nigeria and West Africa, systematic characterization of the indigenous Bacillus isolates of *P. biglobosa* is still limited (Golly *et al.,* 2025).

In the present study, the probiotic properties and antimicrobial activities of the bacterial isolates were then investigated from seeds of *P. biglobosa*, which were traditionally fermented. Emphasis was placed on stress tolerance, molecular identification, antimicrobial effectiveness against clinically important pathogens, and antibiotic susceptibility profiles.

2. Materials and Methods

2.1. Sample collection

The locally processed and sold traditionally fermented seeds of *Parkia biglobosa* (locust bean condiment) were collected from Kuje market (8° 52' 56" N, 7° 13' 13" E), within Kuje Area Council of Abuja, Nigeria. Samples were aseptically taken in sterile polyethylene bags and well labeled for microbiological analysis in the laboratory. The samples were authenticated and botanically identified by a botanist at the Biotechnology Advanced Research Centre, Sheda Science and Technology Complex (SHESTCO). Samples were then immediately processed to limit the alteration of microbial composition. Each fermented seed sample was homogenized in 90 mL of sterile physiological saline solution (0.85% NaCl) in order to get an initial suspension. These were then diluted 10 fold in series with sterile saline solution (ISO 6887-4). Appropriate dilutions were made and 0.1 mL aliquots were spread on de Man–Rogosa–Sharpe (MRS) agar plates, which is a selective culture medium for lactic acid bacteria. The inoculated plates were grown for 24-48 h at 37°C under optimal growth conditions. After incubation, colonies with distinct morphological differences based on such parameters as size, shape, elevation, colour and appearance of the margins were selected and sub-cultured again on fresh MRS Agar plates to obtain a pure culture. The purified isolates were stored on MRS agar slants at 4°C and were subcultured periodically during the study. Pure cultures were given unique identification codes and stored for phenotypic, biochemical, molecular and probiotic assessments (Okolo *et al.,* 2023).

2.2. Phenotypic and Biochemical Characterization

Phenotypic and biochemical methods were used as conventional procedure for preliminary characterization of the bacterial isolates. Initial colony morphology was determined by visual observation of the characteristics of the colonies including pigmentation, texture, elevation, opacity, and edge configuration. Then, cellular morphology was analyzed by Gram staining based on the standard microbiological method. The fresh cultures were smeared and stained to be examined under the oil immersion objective of the microscope to identify the shape, arrangement and Gram reaction of the cells (Dallas & Harrington, 2023).

The catalase activity was assessed by adding a drop of 3% hydrogen peroxide solution to a small amount of bacterial culture on a glass slide. If visible bubbles appeared in the test tube, it was deemed a positive catalase reaction; if no bubbles appeared, it was deemed a negative catalase reaction. Other biochemical tests performed were indole production, methyl red reaction and Voges–Proskauer testing. The assays were carried out using microbiological procedures to obtain metabolic parameters for bacterial identification. The fermentation of glucose, fructose, galactose, lactose, mannitol, starch and sucrose were studied. Individual carbohydrate substrates were added to appropriate fermentation media containing pH indicators. Inoculated tubes were incubated in 37°C and were checked for any colour change and gas production. Positive fermentation reactions were indicated by the production of acid which caused an indicator to change colour. These biochemical tests were performed collectively to give a preliminary identification of the bacterial isolates before molecular confirmation (Cheesbrough, 2006).

2.3 Molecular Identification

The specific isolates were molecularly identified by 16S rRNA gene amplification and sequencing. The overnight cultures of bacteria were used to obtain genomic via the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research Corp., n.d.) extraction protocol according to the manufacturer's instructions. Spectrophotometrically and by agarose gel electrophoresis, the quality and concentration of extracted DNA was examined. The 16S rRNA gene was amplified with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR was carried out in a total volume of 25 µL which contained 12.5 µL of 2× Taq PCR Master Mix (containing Taq DNA polymerase, reaction buffer, dNTPs and $MgCl_2$ at 1.5 mM), 1.0 µL each of forward and reverse primers (10 µM), 2.0 µL of template DNA (50–100 ng) and 8.5 µL of nuclease-free water. Amplification was carried out in a thermal cycler; initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min; and a final extension step at 72 °C for 10 min. The PCR products were kept at 4 °C for further analysis. Amplicons were visualized by electrophoresis on a 1.0% (w/v) agarose gel stained with ethidium bromide (0.5 µg/mL) and run at 100 V for 45 min in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The PCR products were then purified using a Zymo DNA Clean and Concentrator kit, and sequenced at Inqaba Biotech Ltd, Ibadan, Nigeria. The resulting sequences were compared with previously available nucleotide sequences from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). Species identification was performed by sequence similarity and alignment scores with reference strains and sequences validated with biological data (Etuk-Udo *et al.,* 2025).

2.4 Evaluation of Probiotic Properties

Salt Tolerance

Resistance to osmotic stress by the bacterial isolates was determined by growing them in MRS broth media with the addition of various concentrations of sodium chloride (NaCl). Three sets of test media were prepared with NaCl concentration of 2%, 4% and 6%, then the test media were inoculated with standardized bacterial cultures. The inoculated broths were incubated for 24 h at 37°C. Visual observation and OD measurements were used to assess growth. As the microorganisms in fermented foods are often subjected to different osmotic conditions during processing and storage, the ability of the isolates to withstand and grow under high salt levels was deemed as an important attribute of probiotics (Wiśniewski *et al.,* 2025).

Bile Salt Tolerance

Bacterial isolates were grown overnight under aerobic conditions in de Man, Rogosa and Sharpe (MRS) broth at 37 °C for 18–24 h. The cultures were then adjusted to 0.5 ± 0.02, OD 600nm in sterile MRS broth. 100 µL of the standardized culture was inoculated into 10 mL of MRS broth containing 0.3% (w/v) bile salts. The same volume of standardized culture was inoculated in 10mL medium of MRS broth without bile salts (bile-free medium) to prepare control cultures. The test and control cultures were grown under aerobic conditions for up to 6 hours at 37 °C. The growth of the bacteria was monitored spectrophotometrically at one-hour intervals at 600 nm using a UV-visible spectrophotometer. Sterile MRS broth was used as a blank for the absorbance. At the end of the incubation period, 100µL of serial dilutions (10^{-6} to 10^{-8}) were also plated on MRS Agar plates, which were incubated at 37 °C for 48 h and subsequently counted for viable cells. The percentage of the survivors were determined based on the following formula:

$$Survival\ (\%) = \frac{(\text{OD600nm of Test Culture (with bile salt)})}{(\text{OD600nm of Control Culture (without bile salt)})} x\ 100$$

Isolates that exhibited tolerance of ≥ 60% after 6 h exposure to 0.3% bile salt were considered to have good bile salt tolerance (Sudheer *et al.,* 2025).

Temperature Tolerance

Standardized inocula were added to MRS broth and incubated at 20ºC, 37°C, and 45°C. Their growth response was evaluated by optical density measurement at 600 nm and visual observation (turbidity) after incubation. A loopful each was then inoculated on MRS agar plates followed by incubation at 37ºC for 48h. Cell viability was determined via plate count method (Sudheer *et al.,* 2025).

2.5 Antimicrobial Activity

Bacterial isolates were tested for their antimicrobial activity by agar well diffusion method. The pathogens tested were *Escherichia coli, Proteus vulgaris, Salmonella typhi* and *Serratia marcescens*. The fresh cultures of indicator organisms were standardized and the standardized culture was evenly distributed on the surface of Mueller-Hinton agar plates. Wells were aseptically bored into the agar with a sterile cork borer, and the cell-free supernatant was dispensed into the wells from probiotic candidate isolates. The antimicrobial activity was evaluated after 24, 48 and 72 h of incubation and the diameter of the clear zone around the well was measured. The bigger the inhibition zone the greater the antagonistic effect on the test organisms (Shah *et al.,* 2025).

2.6 Antibiotic Susceptibility Testing

Antibiotic susceptibility tests of the isolates were performed by the Kirby–Bauer disc diffusion method. A known number of bacteria were inoculated on each Mueller–Hinton agar plate as standardized suspension, equal to the required turbidity standard. Antibiotic discs (commercial) containing metronidazole (5 µg), tetracycline (30 µg), chloramphenicol (30 µg), and co-trimoxazole (25 µg) were gently placed on inoculated surfaces of the agar with sterile forceps. The plates were incubated at 37°C for 24 hours and then the diameter of the inhibition zones measured in millimeters. All the isolates were classified as susceptible, intermediate, or resistant using the established interpretative guidelines. The antibiotic susceptibility profile gave valuable information on the safety and possible use of the isolates as probiotic cultures (Sudheer *et al.,* 2025).

2.7 Statistical Analysis

All tests were performed in triplicate. One-way analysis of variance (ANOVA) was used to examine the data. A p value < 0.05 was considered statistically significant.

3. Results

3.1 Isolation and Biochemical Characterization of bacteria

Eight bacterial isolates were obtained following cultivation on de Man, Rogosa, and Sharpe (MRS) agar under anaerobic conditions (Figure 1, Table 1). Biochemical characteristics revealed that all isolates were catalase-positive and indole-negative. Isolates 1 and 2 were Gram-negative rods, whereas isolates 3 to 8 were Gram-positive rods. All isolates tested positive for the Voges–Proskauer reaction; all except isolate 2 were methyl red positive. All isolates fermented glucose, fructose, galactose, mannitol, and sucrose. Lactose fermentation was observed only in isolates 1 and 2, whereas starch hydrolysis was restricted to isolates 3 to 8.

3.2 Probiotic Properties of Isolates

3.2.1 Salt Tolerance

All isolates grew at NaCl concentrations of 2%, 4%, and 6% (Table 2). Isolates 3 to 8 demonstrated strong growth at 2% and 4%, and moderate growth at 6%, whereas isolates 1 and 2 showed reduced tolerance at higher concentrations.

3.2.2 Bile Salt Tolerance

All isolates survived in 0.3% bile salts (Table 3). Isolates 3 to 8 exhibited survival rates exceeding 100%, indicating growth under bile conditions, whereas isolates 1 and 2 showed lower survival.

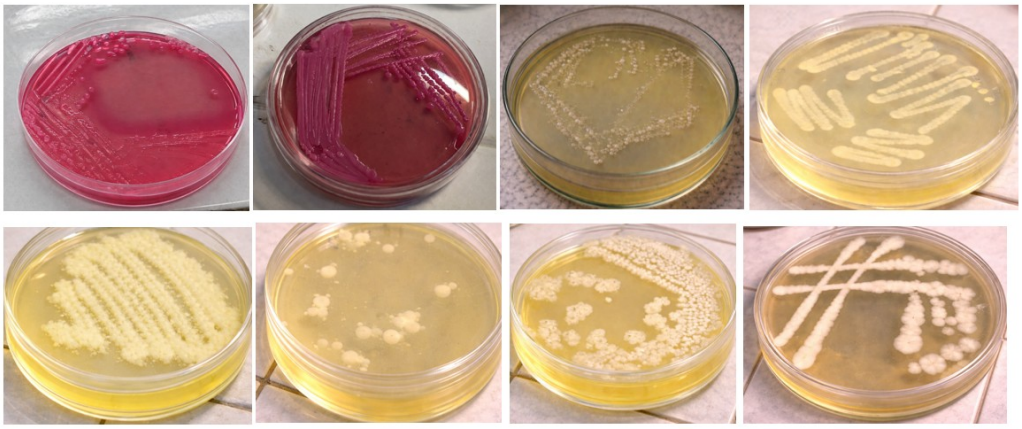

Figure 1. Cross section of bacterial isolates. Top = isolates 1-4, bottom = isolates 5-8.

Table 1. Biochemical Characteristics of Selected Bacterial Isolates

Organism	Gram's	CAT	IND	MR	VR	FRU	GAL	GLU	LAC	MAN	STA	SUC
Sample 1	-ve/rods	+ve	−ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve
Sample 2	-ve/rods	+ve	−ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve
Sample 3	+ve/rods	+ve	−ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve
Sample 4	+ve/rods	+ve	−ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve
Sample 5	+ve/rods	+ve	−ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve
Sample 6	+ve/rods	+ve	−ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve
Sample 7	+ve/rods	+ve	−ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve
Sample 8	+ve/rods	+ve	−ve	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve	+ve

Key: Gram's = Gram Stain, CAT = Catalase, IND = Indole, MR = Methyl Red, VP = Voges Proskauer, FRU = Fructose, GAL = Galactose, GLU = Glucose, LAC = Lactose, MAN = Mannitol, STA = Starch Hydrolysis, and SUC = Sucrose. –ve = negative, +ve = positive

3.2.3 Temperature Tolerance

Optimal growth for all isolates occurred at 37°C (Table 4). Isolates 3 to 8 maintained broader tolerance across temperatures compared to isolates 1 and 2.

Table 2. Salt Tolerance of Bacterial Isolates at Different NaCl Concentrations

Isolate	2% NaCl	4% NaCl	6% NaCl
Sample 1	+++	++	+
Sample 2	+++	++	+
Sample 3	+++	+++	++
Sample 4	+++	+++	++
Sample 5	+++	+++	++
Sample 6	+++	+++	++
Sample 7	+++	+++	++
Sample 8	+++	+++	++

Key: +++ = strong growth; ++ = moderate growth; + = weak growth

Table 3. Bile Salt Tolerance of Bacterial Isolates at 600nm

Isolate	4 h	8 h	12 h	Survival Rate (%)
Sample 1	0.62	0.55	0.48	77%
Sample 2	0.64	0.57	0.50	78%
Sample 3	0.80	0.85	0.88	110%
Sample 4	0.82	0.87	0.90	110%
Sample 5	0.79	0.84	0.87	110%
Sample 6	0.81	0.86	0.89	110%
Sample 7	0.83	0.88	0.91	110%
Sample 8	0.80	0.86	0.90	112%

Key: Survival rate calculated relative to initial OD (4 h). Values > 100% indicate growth in bile conditions.

Table 4. Temperature Tolerance of Bacterial Isolates

Isolate	25°C	37°C	45°C
Sample 1	++	+++	+
Sample 2	++	+++	+
Sample 3	+++	+++	++
Sample 4	+++	+++	++
Sample 5	+++	+++	++
Sample 6	+++	+++	++
Sample 7	+++	+++	++
Sample 8	+++	+++	++

Key: +++ = strong growth; ++ = moderate growth; + = weak growth

3.3 Antimicrobial Activity of Isolates

3.3.1 Activity against *Escherichia coli*

Zones of inhibition at 72 h ranged from 11.10 to 20.30 mm (Table 5). One-way ANOVA showed a significant difference among isolates, $F(7, 16) = 112.45$, $p < 0.001$. Isolates 7 and 8 demonstrated the highest activity.

3.3.2 Activity against *Proteus vulgaris*

Inhibition zones ranged from 10.90 to 20.00 mm (Table 6), with significant variation across isolates, $F(7, 16) = 105.62$, $p < 0.001$.

3.3.3 Activity against *Salmonella typhi*

Zones ranged from 11.00 to 20.80 mm (Table 7), with significant differences observed, $F(7, 16) = 118.33$, $p < 0.001$.

3.3.4 Activity against *Serratia marcescens*

Zones ranged from 10.70 to 20.00 mm (Table 8), with significant variation, $F(7, 16) = 101.87$, $p < 0.001$. Across all test organisms, isolates 7 and 8 consistently exhibited the highest antimicrobial activity.

Table 5. Antimicrobial Activity of Isolates against *Escherichia coli* Using Agar Well Diffusion Method (mm)

Isolate	24 h (Mean ± SD)	48 h (Mean ± SD)	72 h (Mean ± SD)
Sample 1	10.07 ± 0.25	11.20 ± 0.20	11.97 ± 0.15^{e}
Sample 2	9.47 ± 0.25	10.47 ± 0.15	11.10 ± 0.10^{e}
Sample 3	14.00 ± 0.20	15.27 ± 0.25	15.97 ± 0.15^{c}
Sample 4	16.50 ± 0.30	17.97 ± 0.15	18.80 ± 0.20^{b}
Sample 5	14.77 ± 0.25	16.20 ± 0.20	17.00 ± 0.20^{c}
Sample 6	13.23 ± 0.25	14.40 ± 0.20	14.97 ± 0.15^{d}
Sample 7	17.27 ± 0.25	18.77 ± 0.25	19.43 ± 0.20^{a}
Sample 8	18.03 ± 0.25	19.50 ± 0.30	20.27 ± 0.25^{a}

Key: >15 mm = sensitive, 10–15 mm = intermediate, <10 mm = resistant. Values with different superscript letters are significantly different ($p < 0.05$, Tukey's HSD).

ANOVA for *Escherichia coli* activity

Source of Variation	Sum of Squares (SS)	df	Mean Square (MS)	*F*-value	*p*-value
Between Groups	198.64	7	28.38	112.45	< 0.001
Within Groups	4.54	16	0.28		
Total	203.18	23			

Table 6. Antimicrobial Activity against *Proteus vulgaris* (mm)

Isolate	24 h (Mean ± SD)	48 h (Mean ± SD)	72 h (Mean ± SD)
Sample 1	9.20 ± 0.20	10.40 ± 0.20	11.17 ± 0.15^{e}
Sample 2	8.97 ± 0.15	10.17 ± 0.15	10.90 ± 0.10^{e}
Sample 3	13.20 ± 0.20	14.70 ± 0.20	15.40 ± 0.20^{c}
Sample 4	15.77 ± 0.25	17.27 ± 0.25	18.20 ± 0.20^{b}
Sample 5	14.20 ± 0.20	15.70 ± 0.20	16.50 ± 0.20^{c}
Sample 6	12.70 ± 0.20	14.00 ± 0.20	14.70 ± 0.20^{d}
Sample 7	16.77 ± 0.25	18.27 ± 0.25	19.00 ± 0.20^{a}
Sample 8	17.30 ± 0.30	19.03 ± 0.25	20.00 ± 0.20^{a}

Key: >15 mm = sensitive, 10–15 mm = intermediate, <10 mm = resistant. Values with different superscript letters are significantly different ($p < 0.05$, Tukey's HSD)

ANOVA for *Proteus vulgaris* activity

Source of Variation	SS	df	MS	*F*	*p*-value
Between Groups	182.31	7	26.04	105.62	< 0.001
Within Groups	3.95	16	0.25		
Total	186.26	23			

Table 7. Antimicrobial Activity against *Salmonella typhi* (mm)

Isolate	24 h (Mean ± SD)	48 h (Mean ± SD)	72 h (Mean ± SD)
Sample 1	9.70 ± 0.20	11.00 ± 0.20	11.70 ± 0.20^{e}
Sample 2	9.17 ± 0.15	10.40 ± 0.20	11.03 ± 0.15^{e}
Sample 3	13.70 ± 0.20	15.00 ± 0.20	15.70 ± 0.20^{c}
Sample 4	16.23 ± 0.25	17.77 ± 0.25	18.73 ± 0.25^{b}
Sample 5	14.77 ± 0.25	16.23 ± 0.25	17.00 ± 0.20^{c}
Sample 6	13.20 ± 0.20	14.40 ± 0.20	15.03 ± 0.15^{d}
Sample 7	17.20 ± 0.20	18.77 ± 0.25	19.50 ± 0.20^{a}
Sample 8	18.23 ± 0.25	19.77 ± 0.25	20.77 ± 0.25^{a}

Key: >15 mm = sensitive, 10–15 mm = intermediate, <10 mm = resistant. Values with different superscript letters are significantly different ($p < 0.05$, Tukey's HSD)

ANOVA for *Salmonella typhi* activity

Source of Variation	SS	df	MS	*F*	*p*-value
Between Groups	205.78	7	29.40	118.33	< 0.001
Within Groups	3.98	16	0.25		
Total	209.76	23			

Table 8. Antimicrobial Activity against *Serratia marcescens* (mm)

Isolate	24 h (Mean ± SD)	48 h (Mean ± SD)	72 h (Mean ± SD)
Sample 1	9.00 ± 0.20	10.20 ± 0.20	10.97 ± 0.15^{e}
Sample 2	8.70 ± 0.20	9.97 ± 0.15	10.67 ± 0.15^{e}
Sample 3	13.00 ± 0.20	14.40 ± 0.20	15.20 ± 0.20^{c}
Sample 4	15.27 ± 0.25	17.00 ± 0.20	18.00 ± 0.20^{b}
Sample 5	14.00 ± 0.20	15.70 ± 0.20	16.50 ± 0.20^{c}
Sample 6	12.40 ± 0.20	13.70 ± 0.20	14.40 ± 0.20^{d}
Sample 7	16.23 ± 0.25	18.00 ± 0.20	18.83 ± 0.15^{a}
Sample 8	17.23 ± 0.25	19.03 ± 0.25	20.00 ± 0.20^{a}

Key: >15 mm = sensitive, 10–15 mm = intermediate, <10 mm = resistant. Values with different superscript letters are significantly different ($p < 0.05$, Tukey's HSD)

ANOVA for *Serratia marcescens* activity

Source of Variation	SS	df	MS	*F*	*p*-value
Between Groups	176.52	7	25.22	101.87	< 0.001
Within Groups	3.96	16	0.25		
Total	180.48	23			

3.4 Antibiotic Susceptibility Profile

Susceptibility patterns varied across antibiotics (Table 9). Most isolates were resistant to metronidazole, while higher susceptibility was observed for tetracycline and chloramphenicol. Isolates 7 and 8 demonstrated the broadest susceptibility profile across all tested antibiotics.

Table 9. Mean Antibiotic Susceptibility Profile of Bacterial Isolates against selected Antibiotics (mm, ± SD)

Isolate	Metronidazole (30 µg)	Tetracycline (30 µg)	Chloramphenicol (10 µg)
Sample 1	6.50 ± 0.20^{f}	11.80 ± 0.20^{e}	12.50 ± 0.20^{d}
Sample 2	6.20 ± 0.15^{f}	11.20 ± 0.15^{e}	12.00 ± 0.15^{d}
Sample 3	7.80 ± 0.20^{e}	13.50 ± 0.25^{d}	14.80 ± 0.20^{c}
Sample 4	9.50 ± 0.25^{c}	15.80 ± 0.30^{c}	17.20 ± 0.25^{b}
Sample 5	8.80 ± 0.20^{d}	14.20 ± 0.25^{d}	15.80 ± 0.20^{c}
Sample 6	7.50 ± 0.15^{e}	13.00 ± 0.20^{d}	14.50 ± 0.15^{c}
Sample 7	10.20 ± 0.30^{b}	17.50 ± 0.30^{b}	18.80 ± 0.30^{a}
Sample 8	10.80 ± 0.25^{a}	18.20 ± 0.35^{a}	19.50 ± 0.30^{a}

Key: Values are presented as mean inhibition zone diameter ± standard deviation of triplicate determinations (n = 3). Means within the same antibiotic column bearing different superscript letters are significantly different at $p < 0.05$ using Tukey's HSD multiple comparison test. Resistant (R) = <10 mm; Intermediate (I) = 10–15 mm; Sensitive (S) = >15 mm

One-Way ANOVA Summary for Antibiotic Susceptibility Profile

Antibiotic	Between-Group SS	df	Mean Square	F-value	p-value
Metronidazole	27.84	7	3.98	79.60	<0.001
Tetracycline	52.63	7	7.52	104.44	<0.001
Chloramphenicol	61.48	7	8.78	117.07	<0.001
Co-trimoxazole	41.95	7	5.99	96.61	<0.001

3.5 Molecular identification

BLAST analysis identified isolates as members of the genera Klebsiella and Bacillus, with percentage identity ranging from 94.64% to 98.58%, indicating highly significant matches (Table 10).

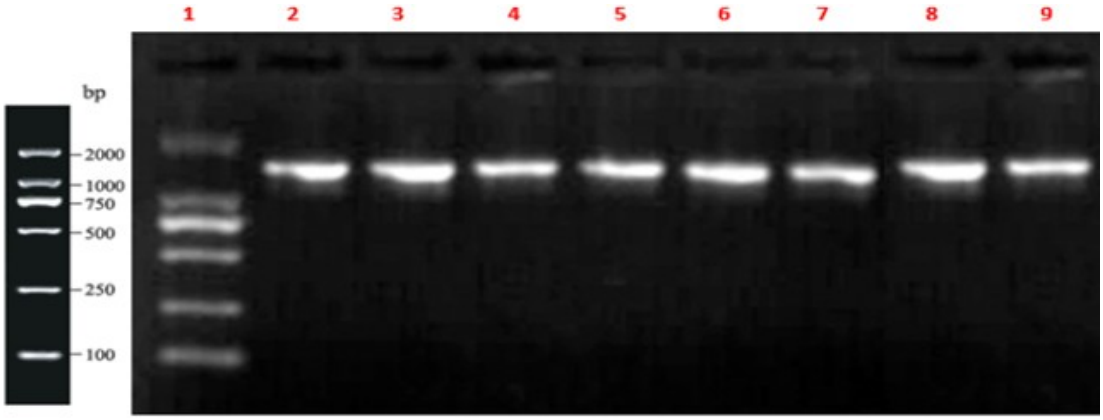

Figure 2. Gel Electrophoresis Micrograph of Amplified gDNA using Polymerase Chain Reaction (PCR). Key: Lane 1= 1kb DNA ladder (MBP Inc), Lane 2 = Isolate 1, Lane 3 = Isolate 2, Lane 4 = Isolate 3, Lane 5 = Isolate 4, Lane 6 = Isolate 5, Lane 7 = Isolate 6, Lane 8 = Isolate 7, Lane 9 = Isolate 8.

Table 10. Molecular Identification via Sanger Sequencing using Basic Local Alignment Search Tool (BLAST) Analysis

Isolate	Description	Max score	Query cover	E-value	Percentage identity (%)	Accession no.	Query length (bp)
1	*Klebsiella pneumoniae* NAMZ6 gene	1007	98%	0.0	94.64	LC843883	628
2	*Klebsiella pneumoniae* strain ERUVET-S72	1725	97%	0.0	97.69	PX760577	1028
3	*Bacillus paramycoides* strain SCAU-184	1879	99%	0.0	97.46	PQ896508	1095
4	*Bacillus subtilis* strain RBM1A	1748	96%	0.0	98.58	OM267754	1215
5	*Bacillus subtilis* strain CA27	1810	100%	0.0	97.36	MT197385	1060
6	*Bacillus velezensis* strain Bac60	1831	99%	0.0	96.48	PQ813794	1108
7	*Bacillus licheniformis* strain C1-5-8	1832	99%	0.0	96.32	KP979476	1114
8	Bacillus *velezensis* strain Bac96	1755	98%	0.0	96.14	PQ813830	1080

4. Discussion

The microbial community isolated from fermented *Parkia biglobosa* was highly dominated by Bacillus species with 75% of the isolates, whereas the rest were *Klebsiella pneumoniae*. This is in line with the microbial ecology of alkaline legume fermentations where the proteolytic members of the bacillaceae predominate as they have the ability to break down proteins and create the alkaline environment necessary to allow the product to mature (Owusu-Kwarteng *et al.,* 2020). Based on biochemical characteristics, the isolates were further divided into Gram-positive Bacillus group and Gram-negative Klebsiella group. There was universal catalase activity among all the isolates, thus providing efficient detoxification of reactive oxygen species, which helps the isolates adapt to the aerobic/facultatively anaerobic environment found in Parkia fermentation (Parkouda *et al.,* 2019). The same applies to the absence of indole production by all the isolates, which is similar to other reports on alkaline fermented condiments, where the absence of tryptophanase activity is conducive to the desirable sensory characteristics due to the reduction of putrefactive metabolites (Harirchi *et al.,* 2022).

The positive Voges-Proskauer test to the isolates indicated that active butanediol fermentation may occur, which favours the production of acetoin and 2,3-butanediol, and lowers the production of acids, thus creating an alkaline environment characteristic of fermented products of locust bean (Mahmud *et al.,* 2023). Conversely, the methyl red reactions were able to differentiate the isolates, with all of the Bacillus species and one *K. pneumoniae* isolate showing positive reactions, while the second Klebsiella isolate was negative, indicating strain variation in glucose metabolism that has been reported previously in the species (Abid *et al.,* 2022). The ability to metabolise a wide range of carbohydrates, such as glucose, fructose, galactose, mannitol and sucrose, shows a high level of metabolic flexibility, allowing for efficient use of the variety of carbohydrate fractions found in the seeds of *P. biglobosa* (Murakami *et al.,* 2023). On the other hand, Klebsiella was the sole genus to ferment lactose and Bacillus the sole genus to hydrolyse starch, indicating the presence of extracellular α-amylase which is a polysaccharide degrading enzyme secreted during fermentation (Ganesh *et al.,* 2024; Prongjit *et al.,* 2025).

The molecular identification of the 16S rRNA gene sequencing of isolates previously identified as *Enterobacter spp.* as *Klebsiella pneumoniae* confirmed the biochemical identification and revealed that this method is limited in distinguishing closely related Enterobacteriaceae, underscoring the necessity of molecular identification in the screening of probiotics (Adesulu-Dahunsi *et al.,* 2025). The presence of other species, such as *Bacillus paramycoides, B. subtilis, B. velezensis*, and *B. licheniformis*, further bolsters the notion of the Bacillus consortium that is commonly found in spontaneous alkaline fermentation, which has species occupying complementary ecological niches based on their enzymatic functions and environmental adaptations (Obafemi *et*

al., 2024). Despite some sequence divergence seen for one Klebsiella isolate, these taxonomic assignments are robust, with high sequence identity (94.64–98.58%) and query coverage (96–100%) and E-values of 0.0 supporting the strength of these taxonomic assignments.

The probiotic potential of isolates was significantly higher for the Bacillus isolates compared to Klebsiella isolates based on functional evaluation. The tolerance to 6% NaCl is indicative of efficient osmoadaptive mechanisms such as compatible solute accumulation, membrane modification and ion transport that will be required to ensure survival under gastrointestinal osmotic stress (Evangelista *et al.,* 2024; Payne *et al.,* 2024). Likewise, Bacillus isolates continued to grow in 0.3% bile salts with greater than 100% survival, while Klebsiella showed moderate survival. This improved resistance to bile has been thought to be due to the activity of bile salt hydrolase, adaptation of the cells to the actual concentration of bile, the activity of efflux pumps and stress-response proteins, all of which are involved in the tolerance or growth of the cells during bile concentrations present in the physiological range (Jin *et al.,* 2026; Borthakur *et al.,* 2026). Additionally, the wide range of temperature tolerance observed in the Bacillus strains, especially their ability to grow at 45°C, indicates thermotolerance and spore-forming abilities, which enhance the stability of the products and ensure their survival in the gastrointestinal tract (Liao *et al.,* 2025). Together, these results confirm the ability of Bacillus isolates to resist stress conditions and their beneficial physiological characteristics as potential probiotics, while the comparatively low stress tolerance and opportunistic nature of *K. pneumoniae* make it less suitable for probiotic applications (Obafemi *et al.,* 2024).

The antimicrobial property also highlighted the probiotic potential of recovered isolates, with the *Bacillus velezensis* and *B. licheniformis* strains showing the highest and wide range of inhibitory activity against *Escherichia coli, Proteus vulgaris, Salmonella typhi* and *Serratia marcescens*. The progressive zone enlargement during the incubation period suggests that antimicrobial metabolites are continuously being produced during the late exponential and stationary growth periods, which is a common feature of the secondary metabolism of Bacillus. The inhibition diameters ranged from 18.80 mm to 20.80 mm, which is greater than the accepted susceptibility threshold, and is similar to the one reported for established probiotic strains, indicating the remarkable antagonistic capability of the isolates (Hu *et al.,* 2025). This activity is likely due to synergic production of antimicrobial compounds (bacteriocins and lipopeptides (surfactin, fengycin, iturin), as well as bioactive metabolites (bacillaene, difficidin, macrolactin, bacillibactin) that show broad-spectrum antibacterial activity (Markelova & Chumak, 2025). Organic acid production might also play a role in pathogen inhibition, but it is important to identify if there are peptide specific antimicrobial properties by further investigation with neutralised culture supernatants (Nicolas, 2025).

The antagonistic activities of *Klebsiella pneumoniae* were relatively low, which is similar to other studies reporting *K. pneumoniae's* lower antimicrobial metabolite production compared to Bacillus species (Sharma *et al.,* 2024). The resistance pattern found for *E. coli* and *S. typhi* and the slightly lower resistance of *P. vulgaris* and *S. marcescens* is likely due to species-specific resistance mechanisms such as efflux pumps, membrane permeability barriers and enzymatic detoxification systems (Zhang *et al.,* 2024). In addition, the antibiotic susceptibility profiles showed that the Bacillus isolates were superior. The *Bacillus spp*. remained susceptible to tetracycline, chloramphenicol and co-trimoxazole while Klebsiella spp. had reduced susceptibility or resistance to these clinically important antibiotics, which was not surprising as metronidazole is selective against obligate anaerobes (Sharma *et al.,* 2024; Tasew *et al.,* 2025). This susceptibility is favourable for probiotic candidates since it reduces the chance of being sources from which to transfer antibiotic resistance genes. However, before commercialization, it is still necessary to conduct safety evaluation (including haemolytic activity, virulence assessment and expanded antibiotic susceptibility profiling (Zhang *et al.,* 2024).

Overall, the data obtained revealed that fermented *P. biglobosa* serves as an ideal source of probiotic bacteria strains, adaptable for gastrointestinal stress, with versatility against a spectrum of disease-causing bacteria. Future investigations pertaining to whole-genome sequencing, safety assessment, adhesion to intestinal epithelial cells, and *in vivo* validation of such probiotic bacteria should be assessed to confirm their efficacy and safety for human consumption.

5. Conclusion

The traditionally fermented seeds of *Parkia biglobosa* are rich in various bacterial populations with a predominance of probiotic species of the genus Bacillus. *Bacillus paramycoides, B. subtilis, B. velezensis,* and *B. licheniformis* were identified as major bacterial components of the microbial community based on molecular analyses. Both the isolates showed good tolerance with respect to osmotic, bile and temperature stress and

revealed high level of probiotic potential. Moreover, the ability of *B. velezensis* and *B. licheniformis* to show pronounced antimicrobial activity against major enteric pathogens and their antibiotic susceptibility profiles were favourable. The results indicate fermented product of *P. biglobosa* as a good source of indigenous probiotic microorganisms having potential applications in functional foods, bio-preservation and additional antimicrobial therapy applications with relevance to cultural preservation. These strains should be developed as commercial probiotic strains and future studies should be directed towards *in vivo* validation and safety evaluation as well as towards whole genome sequencing.

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