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Probiotic Capability of Bacillus spp. Isolated from Iru - Fermented African Locust Bean (Parkia biglobosa)

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Abstract

Bacillus species have diverse applications in agriculture, enzyme production and medicine to produce vaccines and probiotics. Currently, little is known about the probiotic potential of *Bacillus* from fermented condiments of African origin. This work aimed at isolating *Bacillus* spp. with probiotic potential from fermented African locust bean - *iru*. *Iru* samples were obtained from a local market in Oyo, Nigeria. The samples were pretreated by boiling to eliminate vegetative cells and inoculated using pour plate technique. Isolates were identified; their antibiotic susceptibility profile and antimicrobial activity against known pathogens (*Listeria monocytogenes, Salmonella enterica* and *Staphylococcus aureus*) were determined. Safety and technological properties (cellulase, protease, lipase and amylase activities) of the isolates were also determined. Twenty spore-forming, Gram-positive with rod shape were isolated and identified as *Bacillus subtilis* (8), *Bacillus licheniformis* (4), *Bacillus pumilus* (3), *Bacillus polymyxa* (1), *Bacillus licheniformis* (1), *Bacillus alvei* (1), *Bacillus badius* (1) and *Bacillus* sp. (1). All isolates were susceptible to levofloxacin, ciprofloxacin, ofloxacin, gentamycin and azithromycin while 16 (80%) were resistant to cefotaxime. Only 11 (55%) isolates inhibited the growth of *Listeria monocytogenes,* with no inhibition recorded against other pathogens. The 11 isolates synthesized at least one enzyme, with *B. subtilis* PA1 and *B. alvei* PB5 producing the four enzymes determined. γ-haemolysis was exhibited in 90% of the isolates while 10% exhibited α-haemolysis. *Bacillus subtilis* PA1, *Bacillus subtilis* PA6 and *Bacillus licheniformis* PA5 survived high acid, bile and simulated acid and bile. *Bacillus subtilis* and *Bacillus licheniformis* from African fermented locust beans have excellent probiotic potentials.

Keywords: *Acid and Bile Tolerance; Antimicrobial Activity; Enzymes; Probiotic Potential; Safety Properties*

Introduction

Iru is a popular indigenous fermented condiment obtained from the seeds of African locust beans (*Parkia biglobosa*). It is a popular cooking ingredient among the Yoruba people of Nigeria, and it serves as a flavor enhancer for soups and stews. *Iru* is also well-known in many West African countries [1]. The fermented locust bean is a high-nutritional-value food that contains protein, fat, fiber, and carbohydrates. It is an excellent source of calcium and fat, particularly for rural dwellers, and it also serves as a low-cost protein supplement in the diets of poor families due to its high protein content [2]. The unfermented or natural locust beans are not usually consumed due to the presence of carbohydrates, some of which include raffinose, stachyose, and arabinogalactan which are generally undigestible and also due to the presence of various anti-nutritional factors which make it generally unfit for consumption [3].

Fermentation of the African locust beans consequently is important and necessary because it helps increase nutritional value, improves shelf-life, aids digestibility, and enhances organoleptic properties like taste, texture, and flavour. It also enhances its enzymatic activity [4]. Because raw African locust beans are nutritionally deficient and unpleasant to consume, the physical, chemical, biochemical, and nutritional properties of the African locust bean seeds change instantly after fermentation. [2]. *Iru* is a product of alkaline fermentation [3]. *Bacillus* spp. mainly *B. subtilis* have been discovered to be part of the most predominant organisms found in *iru* responsible for fermentation [4]. During fermentation, they hydrolyze proteins into amino acids and ammonia, resulting in an increase in pH. [2]. *Bacillus* species have a wide range of appealing applications, including the production of enzymes, secretory proteins, antimicrobial compounds, vitamins, and carotenoids among others [5]. They also have increased tolerance and survivability in the gastrointestinal tract, which has raised the probiotic candidature of some *Bacillus* species [6].

Probiotics are living organisms that, when administered in sufficient quantities, provide health benefits to the host [7]. Probiotic strains can be found in a variety of dairy foods, dietary supplements, and fermented foods and are intended to provide a variety of health benefits in humans. Probiotics have been used to prevent gastrointestinal infections in animal feed, and they are also widely used in the poultry and aquaculture industries [8].

Aside from the commonly studied strains (Lactic acid bacteria - LAB and Bifidobacterium), the majority of the *Bacillus* genus possesses probiotic properties. Compared to LAB, however, *Bacillus* species have not gotten widespread popularity, particularly in the area of research interest. Several *Bacillus* species and strains have been tested *in vitro* and *in vivo* for potential probiotic abilities. They are more acid-tolerant and more stable during variety of temperature processing and storage. They have also been reported to have antioxidant, antimicrobial, enzyme production, and food fermentation properties [9]. Despite the benefits listed above, these strains have not received much traction in the functional food industry because of their similarity to a few human pathogens such as *B. anthracis, B. cereus,* and others [8].

Probiotic strains' health benefits are primarily determined by their ability to overlive passage through the upper gastrointestinal tract, colonize, replicate and multiply in the host intestine. A sufficient number of the viable probiotic bacteria must enter the target site in the host's system [10]. There is little information on the probiotic potential of *Bacillus* species isolated from African fermented locust bean; therefore, the aim of this study is to evaluate the probiotic properties of *Bacillus* species isolated from 'iru'.

Materials and Methods

Sample collection and isolation of *Bacillus* spp

Iru samples were collected from a local market in Oyo town, Oyo State, Nigeria. The samples were pretreated by boiling at 100°C for 7 - 8 minutes in McCartney bottles to eliminate all the vegetative cells. The preheated samples were weighted (1g), and transferred into a sterile stomacher bag (SEWARD, UK) and homogenized using a stomacher (STOMACHER 80 BIOMASTER, SEWARD, UK) with 9 mL sterile water for 2 min to obtain a homogenate. The homogenate was serially diluted using sterile water and pour plated on nutrient agar in duplicates, the plates were then incubated at 37ºC for 24h.

Identification of isolates

All isolates obtained from this study were identified using cultural, morphological characteristics and biochemical tests following Bergey's manual.

Safety aspect of selected strains

Antibiotic susceptibility of isolates

The Kirby-Bauer disc diffusion method was used, following the Clinical and Laboratory Standards Institute [11] standard. The antibiotic susceptibility pattern of the isolates was tested using the Gram-positive specific antibiotic sensitivity discs (CELTECH DIAGNOSTICS, BELGIUM) containing twelve different antibiotics which are imipenem/cilastatin (10/10 µg), cefuroxime (30 µg), ofloxacin (5 µg), erythromycin (15 µg), gentamycin (10 µg), azithromycin (15 µg), cefotaxime (25 µg), amoxicillin-clavulanate (30 µg), ceftriaxone sulbactam (45 µg), cefixime (5 µg), levofloxacin (5 µg) and ciprofloxacin (5 µg). Six groups of antibiotics were investigated: carbapenem, cephalosporin, penicillin, fluoroquinolone, macrolide, and aminoglycosides. The diameter of zones of inhibition around the discs was measured using a meter rule to the nearest millimetre and interpreted according to the CLSI standard [11].

Antimicrobial activity of isolates against pathogens

The isolate metabolites were evaluated for their antagonistic activity against some gastrointestinal pathogenic bacteria which include *Staphylococcus aureus*, *Listeria monocytogene*s and *Salmonella enterica* using the agar well diffusion method. The isolates were inoculated into Nutrient broth and incubated at 37°C for 24h. The broth was aseptically transferred into sterile test tubes and were centrifuged at 4000 rpm (revolutions per minute) for 15 minutes. The antimicrobial effect of the supernatant of each isolate was tested against the pathogens. The antimicrobial effects were recorded by measuring the zone of inhibition around the wells using a meter rule [11,12].

Hemolytic activity

The haemolytic assay was carried out using freshly prepared blood agar (5% antibiotic-free fresh blood in Nutrient Agar). A single line of the streak was made across the plate and incubated at 37°C for 24h. The presence of a clear zone around the line of streak indicates lysis of the blood cells by the isolates indicating Beta haemolysis; appearance of a greenish or brown colour change around the line of streak without a clear zone confirms Alpha haemolysis while Gamma haemolysis results as a result of no change on the agar surface [13].

Biofilm synthesis

Biofilm formation among the isolates was detected qualitatively using the method of Chaleb., *et al*. (2007). Isolates were radially streaked on Nutrient agar supplemented with 4% Congo red dye. The plates were incubated at 37°C for 24 to 48h. Formation/presence of black colonies on Congo red agar indicates ability of the organism to produce biofilm while inability of the organism to produce biofilm is indicated by the formation of pink colonies [14].

Ability to withstand simulated gastrointestinal (GIT) condition

Preparation of inocula

Young culture of isolates were subcultured into 10 mL Nutrient Broth, and incubated at 37°C for 24h. The broth was transferred into sterile tubes and were centrifuged at 4000 rpm for 15 minutes and the pellets/cells were re-suspended in sterile water and standardized to 0.5 McFarland.

Acid tolerance test

The method used by Klingberg., *et al*. [15] was used with modifications. Nutrient Broth was adjusted with Hydrochloric acid (HCl) 1N to obtain a final pH of 2.5. For each isolate, 0.1 mL of the standardized inoculum was added into 10 mL of Nutrient Broth which has been

adjusted to pH 2.5 and incubated at 37°C. The survival of each isolate at acidic pH (2.5) was determined at four incubation periods (0, 1, 2, 3, and 4 hours). The initial and final pH were recorded. The acid tolerance was estimated by the viable cell count in all Nutrient Agar plates. The survival rate was calculated using the formula:

Survival rate (%) = (log CFU N_t/log CFU N_0) × 100

Where N_t represent the surviving viable count after 1, 2, 3, 4h

N_0 represent the initial (0 h) viable count [16].

Tolerance to bile salts

The method of Klingberg., *et al*. [15] was modified to determine bile salts tolerance of isolates. Nutrient broth was supplemented with 0.3 % bile salt. For each isolate, 0.1 mL of the standardized inoculum was added into 10 mL of nutrient broth already supplemented with bile and incubated at 37°C. The survival of each isolate in bile salt was determined at four incubation periods (0, 1, 2, 3 and 4 hours). The initial and final pH were recorded. The bile salt tolerance was estimated by the viable cell count in all nutrient agar plates. The survival rate was calculated as earlier stated.

Tolerance to simulated acid and bile

In a bid to mimic the parameters present in the gastric environment, isolates that can survive up to 4 hours in both acid and bile were tested further in nutrient broth adjusted to pH 2.5 and also supplemented with 0.3 % bile. For each isolate, 0.1 mL of the 0.5 McFarland standardized inoculum was added into 10 mL of nutrient broth already supplemented with bile and adjusted with Hydrochloric acid (HCl) 1N to obtain a final pH of 2.5 and incubated at 37°C. The survival of each isolate at acidic pH (2.5) and in bile salt was determined at four incubation periods (0, 1, 2, 3, and 4 hours). The initial and final pH was recorded. The acid and bile salt tolerance was estimated by the viable cell count in all Nutrient Agar plates. The survival rate was calculated as earlier stated.

Technological aspect of strains

The twenty (20) isolates were qualitatively screened for enzymes (cellulase, amylase, lipase and protease) production. Isolates with ability to produce these enzymes were further assayed quantitatively for their enzymatic activities.

Cellulolytic activity

A fresh culture of each test organism was inoculated in a broth containing carboxymethyl cellulose (CMC) and incubated at 37ºC for 24h. The cells were harvested/collected by centrifugation at 10,000 rpm for 15 min and the supernatant served as a source of crude enzyme solution. The amount of reducing sugars liberated during hydrolysis was used to determine cellulase activity using the DNS (3,5-dinitro salicylic acid) method described by Farjana and Narayan [17]. One percent carboxymethyl cellulose (CMC) in 1 M citrate buffer (pH 5.0) was used as a substrate. 0.5 mL of the crude enzyme (supernatant) was added into 0.5 mL of 1% CMC. The mixture was incubated at 45ºC for 30 minutes. 1 mL of DNS was added to the solution and the mixture was brought to boiling in a water bath for 10 minutes to stop the reaction [17]. The mixture was allowed to cool in water for colour stabilization and the optical density was read at 540 nm using a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 micromole of glucose within 1 min of reaction.

Proteolytic activity

Each test organism was inoculated into a production medium (0.05g glucose, 0.075g peptone, and salt solution; 5g $MgSO_4$ and 0.05g KH_2PO_4) and incubated at 37ºC for 72h in a shaking incubator (140 rpm). After fermentation, the fermentation broth was centrifuged at 10,000 rpm at 4ºC for 15 minutes and the supernatant was used as a crude enzyme solution. The protease activity was assessed according to Folashade and Joshua [18] by incubating 1 mL of azocasein in Tris-HCl buffer with 1 mL of enzyme solution for 60 minutes at 37ºC. The reaction was stopped by adding 0.5 mL of Trichloroacetic acid (TCA) and was shaken. This solution was left for 15 minutes before being centrifuged at 4ºC for 15 minutes at 3000 rpm, after which 1 mL of the supernatant was added to 1 mL of NaOH (1 M) and absorbance was read at 440 nm. Under standard assay conditions, one unit of protease activity was defined as one micromole of substrate converted per minute.

Amylolytic activity

A loopful of each isolate was inoculated on nutrient broth and incubated at 37ºC for 24h. The cell was harvested by centrifugation at 10,000 rpm for 15 min and the supernatant was used as a source of crude enzyme. Amylase activity was carried out by the 5-Dintirosalicylic acid (DNSA) method according to Okorie and Olasupo [19] with certain modifications. One milliliter (1 mL) of 1 % starch solution was added to 1 mL of the crude enzyme and incubated in a water bath at 55ºC for 15 minutes. Thereafter, 2 ml of DNSA was added to it and was brought to boiling in water bath for 10 minutes to stop the reaction. The solution was allowed to cool in water for colour stabilization and the optical density was determined using a spectrophotometer at 540 nm. Under assay conditions, one unit of enzyme activity was defined as the amount of enzyme required to catalyze the liberation of reducing sugar equivalent to 1 micromole of D-glucose per minute.

Lipolytic activity

Lipase isolation was carried out by submerged fermentation following the method described by Veeresh., *et al.* [20] with few modifications. A loopful of each test organism was transferred into 5 mL of nutrient broth supplemented with peptone (0.05g), $NaNO_3$ (0.001g) and $MgSO_4$ (0.001g) and incubated at 37ºC in a rotary shaker for 24h. One milliliter of the inoculum was inoculated into 50 mL of the production medium. It was incubated for 24h at 37ºC and agitated at 100 rpm in a rotary shaker. The fermented broth was centrifuged at 10,000 rpm for 20 minutes and the supernatant was used as a crude enzyme source. Lipolytic activity was determined by the titrimetric method as described by Kalpana., *et al.* [21] with modifications using olive oil as substrate. Four grams of gum Arabic was emulsified in 8 mL of olive oil and sodium phosphate buffer (0.2M; pH 7.0) to make up to 80 mL. A hundred microlitres of the crude enzyme was added to the emulsion and incubated for 15 minutes at 37ºC. The reaction was stopped and fatty acids were extracted by addition of 1.0 mL acetone: ethanol solution (1:1). The amounts of fatty acids liberated were estimated by titrating with 0.05M NaOH using phenolphthalein as an indicator.

Results and Discussion

Isolation and identification

Twenty Gram positive, rod-shaped, aerobic and endospore forming bacteria were isolated from *iru* samples and were identified as *Bacillus subtilis* (8), *Bacillus licheniformis* (5), *Bacillus pumilus* (3), *Bacillus polymyxa* (1), *Bacillus badius* (1), *Bacillus alvei* (1), and *Bacillus* sp. (1) (Table 1). The presence of *Bacillus* species in *iru* sample could be attributed to their involvement in the fermentation process as reported by Olanbiwoninu., *et al* [3].

S/N	Isolate Code	Gram Reaction	Endospore	Swollen Spore	Cell diameter ≥ 1µm	Catalase	VP	Citrate	Starch	6.5% NaCl growth	Growth at 55ºC	Glucose	Mannitol	Maltose	Lactose	Probable Identity
1	PA1	+	+	+	-	+	+	+	+	+	-	+	+	+	+	*Bacillus subtilis*
2	PA2	+	+	-	-	+	+	-	-	+	-	+	+	+	-	*B. pumilus*
3	PA3	+	+	-	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
4	PA4	+	+	-	-	+	+	+	+	+	+	+	-	+	-	*B. licheniformis*
5	PA5	+	+	+	-	+	-	-	+	+	+	+	+	+	+	*B. licheniformis*
6	PA6	+	+	+	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
7	PA7	+	+	-	-	+	+	+	+	+	+	-	-	+	+	*B. licheniformis*
8	PA8	+	+	-	-	+	+	+	+	+	+	-	+	+	+	*B. licheniformis*
9	PA9	+	+	-	-	+	-	+	-	+	-	-	-	+	+	*Bacillus* sp.
10	PA10	+	+	+	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
11	PB1	+	+	-	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
12	PB2	+	+	-	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
13	PB3	+	+	-	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
14	PB4	+	+	-	-	+	-	-	+	+	-	+	-	+	+	*B. badius*
15	PB5	+	+	-	-	+	+	-	+	+	-	-	-	-	-	*B. alvei*
16	PB6	+	+	+	-	+	+	-	-	+	-	+	+	+	+	*B. pumilus*
17	PB7	+	+	-	-	+	+	-	+	-	-	-	+	+	-	*B. polymyxa*
18	PB8	+	+	+	-	+	+	+	+	+	-	+	+	+	+	*B. subtilis*
19	PB9	+	+	-	-	+	+	+	+	+	+	+	+	+	-	*B. licheniformis*
20	PB10	+	+	+	-	+	+	-	-	+	-	+	+	+	+	*B. pumilus*

Table 1: *Biochemical identification of isolates.*

Safety aspect of selected strains

Antibiotic susceptibility of isolates

The antibiotic susceptibility carried out on the isolates is an important criterion as it ensures that the organisms selected for use as probiotics are not resistant to antibiotics which may cause more harm than good when administered to the patient. The susceptibility pattern of *Bacillus* isolates is as shown in figure 1. All isolates exhibited full susceptibility to levofloxacin, ciprofloxacin, ofloxacin, gentamycin, and azithromycin (fluoroquinolones) while the highest resistance (80%) was against Cefotaxime (a cephalosporin under the β-Lactam group). This may be due to the reduced permeation of the drug through the outer membrane of the bacteria or the inactivation of the antibiotic by β-lactamase, an enzyme that inhibits the activity of β-lactam [22].

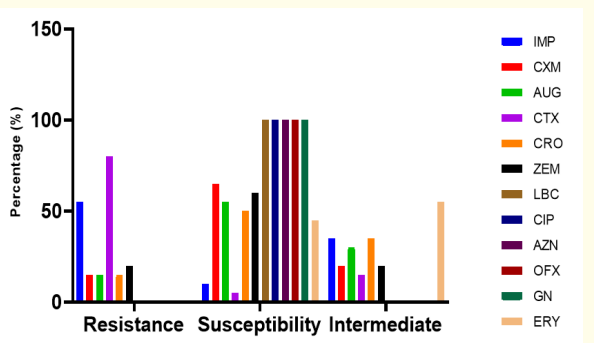

Figure 1: *Antibiotics susceptibility of Bacillus species isolated from 'iru' to antibiotics.*

Key: IMP: Imipenem/cilastatin, CXM: Cefuroxime, AUG: Amoxicillin Clavulanate, CTX: Cefotaxime, CRO: Ceftriaxone sulbactam, ZEM: Cefixime, LBC: Levofloxacin, CIP: Ciprofloxacin, AZN: Azithromycin, OFX: Ofloxacin, ERY: Erythromycin, GN: Gentamycin.

Antimicrobial activity of isolates against pathogens

The results of antimicrobial activity carried out to test whether the strains can be used as biological agents against food spoilage organisms is shown in table 2. About 55% [11] of the tested strains showed activity against only one of the three food-borne pathogens used which is *Listeria monocytogenes.* This demonstrates the ability of some *Bacillus* spp. to synthesize antimicrobial and inhibitory compounds such as organic acids with a broad spectrum of action and antimicrobial proteins known as bacteriocins. Bacteriocins are proteins or protein complexes with bactericidal activity against closely related species to the producer species [23]. These results suggest the possible use of the selected *Bacillus* species in food to reduce the chances of *Listeria* infection. Metabolites produced by *Bacillus* species are used as antimicrobial agents, food preservatives, or bio preservatives, and they are being researched for use in the development of newer therapeutics and antibiotics to fight infections [23]. The eleven *Bacillus* species that exhibited antimicrobial activity were selected for further study.

Serial No.	Isolate Code and Identity	*Salmonella enterica*	*Staphylococcus aureus*	*Listeria monocytogenes*
1	*Bacillus subtilis* PA1	0	0	20
2	*B. pumilus* PA2	0	0	18
3	*B. subtilis* PA3	0	0	20
4	*B. licheniformis* PA4	0	0	8
5	*B. licheniformis* PA5	0	0	12
6	*B. subtilis* PA6	0	0	8
7	*B. licheniformis* PA7	0	0	0
8	*B. licheniformis* PA8	0	0	0
9	*Bacillus* sp.PA9	0	0	0
10	*B. subtilis* PA10	0	0	0
11	*B. subtilis* PB1	0	0	12
12	*B. subtilis* PB2	0	0	18
13	*B. subtilis* PB3	0	0	20
14	*B. badius* PB4	0	0	10
15	*B. alvei* PB5	0	0	10
16	*B. pumilus* PB6	0	0	0
17	*B. polymyxa* PB7	0	0	0
18	*B. subtilis* PB8	0	0	0
19	*B. licheniformis* PB9	0	0	0
20	*B. pumilus* PB10	0	0	0

Table 2: *Antimicrobial activities (mm) of Bacillus species isolated from 'iru' against selected test organisms.*

Haemolytic activity

For food safety to be ensured, according to the European Food Safety Authority (EFSA), evaluation of haemolytic activity is highly recommended and essential if the isolated strain is intended to be used in food products. In this study, the haemolytic activities of eleven selected isolates were evaluated on blood agar plates. Ten of the tested strains showed γ-haemolytic activity while one showed α-haemolytic activity. This is similar to the report of Iqra., *et al.* [24] that all eight evaluated strains showed γ-haemolytic activity which is a supporting

property for their safety. Haemolytic activity is a very important factor to consider when the safety of intended probiotic strains is concerned because such strains are non-pathogenic and non-virulent and also lack haemolysin, a substance that lyses red blood cell membranes [25]. Beta (β)-haemolysis is considered harmful while alpha (α) and gamma (γ) haemolysis are considered safe [26]. This means all the eleven selected strains can be regarded as safe.

Biofilm synthesis

All selected *Bacillus* spp. were unable to synthesize biofilm and this is a food safety advantage. This is because biofilms are associated with high levels of antibiotic resistance, toxins can also be secreted by biofilms found in food which may lead to individual or multiple foodborne intoxication cases. The presence of biofilms in food or food processing plants generally put human health at risk [27].

Ability to withstand simulated gastrointestinal (GIT) conditions

Acid tolerance

The probiotic potential of the species tested further by checking their ability to survive in acid helps to study the survival of different species under the low pH condition of the gastric juice, the pH used in this study was 2.5. The result obtained from the ability of isolates to survive low pH 2.5 at 0, 1, 2, 3 and 4h is presented in table 3. Only four isolates were able to survive, more specifically *Bacillus subtilis* PA1 showed the highest tolerance to this pH for up to 4h with a survival rate of 98.99%. The survival rates showed high variability (98.99% - 45.38%) which suggests that acid tolerance is species/strain specific. This result is consistent with that of Lim., *et al*. [28] who recorded survival rate of 97.45% in acid at 3h of incubation. Decrease in pH of medium was observed in almost all the isolates except medium where *B. licheniformis* PA5 was grown that increased from initial 2.5 to 2.6 after 4 hours.

S/N	Isolate code and identity	Survival rate (%)				pH	
		1h	2h	3h	4h	0h	4h
1	*B. subtilis* PA1	78.99	98.45	98.99	98.99	2.5	2.3
2	*B. pumilus* PA2	60.59	NG	NG	NG	2.5	1.5
3	*B. subtilis* PA3	51.60	45.38	NG	NG	2.5	2.5
4	*B. licheniformis* PA4	81.94	71.17	NG	NG	2.5	2.0
5	*B. licheniformis* PA5	96.07	82.22	81.81	71.57	2.5	2.6
6	*B. subtilis* PA6	84.67	85.45	82.89	81.87	2.5	2.5
7	*B. subtilis* PB1	NG	NG	NG	NG	2.5	1.6
8	*B. subtilis* PB2	82.71	68.74	66.78	NG	2.5	1.8
9	*B. subtilis* PB3	NG	NG	NG	NG	2.5	2.5
10	*B. badius* PB4	89.31	85.15	79.90	73.61	2.5	2.5
11	*B. alvei* PB5	59.45	NG	NG	NG	2.5	2.4

Table 3: *Acid resistance of selected Bacillus species isolated from 'iru'.*

Key: NG - No growth.

Tolerance to bile salts

For bacteria to function as a probiotic, it has to be able to grow and survive in bile which is an important component of the small intestine. Bile concentration of 0.3% has been recommended as suitable for selecting probiotics and the same concentration was used in

this study. From table 4, nine out of the eleven selected *Bacillus* spp. showed great tolerance to bile salts and were able to grow for up to 4h. This result is slightly different from that of Donatien., *et al*. [29] who reported that all tested isolates exhibited excellent tolerance to 0.3% bile salt. *Bacillus licheniformis* PA4 had highest survival rate (129.97%) after four hours of incubation. The survival rate was very high, ranging from (129.97% - 55.02%). There was increase in the pH of media where *B. badius* PB4, *B. subtilis* PB3, *B. subtilis* PA6 and *B. licheniformis* PA5 and *B. subtilis* PA1 were incubated to determined bile tolerance from 7.5 to 8.0, 7.7, 7.6, 7.8 and 7.7, respectively while decrease in pH was observed in others.

S/N	Isolate code and identity	Survival rate (%)				pH	
		1h	2h	3h	4h	0h	4h
1	*B. subtilis* PA1	99.27	99.0	100	100.23	7.5	7. 7
2	*B. pumilus* PA2	84.74	89.24	83.34	62.44	7.5	6.8
3	*B. subtilis* PA3	93.32	95.58	87.68	86.50	7.5	7.2
4	*B. licheniformis* PA4	88.24	93.54	94.27	129.97	7.5	7.0
5	*B. licheniformis* PA5	99.35	98.63	93.86	93.07	7.5	7.8
6	*B. subtilis* PA6	99.04	84.56	93.86	95.79	7.5	7.6
7	*B. subtilis* PB1	83.33	83.33	NG	NG	7.5	7.0
8	*B. subtilis* PB2	63.20	55.02	NG	NG	7.5	7.2
9	*B. subtilis* PB3	97.92	92.36	86.66	86.49	7.5	7.7
10	*B. badius* PB4	99.77	89.21	93.86	99.54	7.5	8.0
11	*B. alvei* PB5	>100	>100	>100	>100	7.5	6.6

Table 4: *Bile tolerance of selected bacillus strains isolated from 'iru'.*

Key: NG - No growth.

Tolerance to simulated acid and bile

In an attempt to imitate the gastric environment, the four isolates (*Bacillus subtilis* PA1 and PA6, *Bacillus licheniformis* PA5, *Bacillus badius* PB4) that survived up to four hours in both acid and bile were selected for further studies and grown in broth containing a mixture of 0.3% bile and acid (HCl) 1N adjusted to pH 2.5. Three of the four isolates were able to survive for up to four hours which makes them good probiotic candidates as shown in table 5. Highest microbial load was observed in the medium where *Bacillus subtilis* PA1 was grown throughout the four hours of investigation. There was increase in the pH of the growth media of the four isolates after four hours of incubation.

S/N	Isolate code and identity	Survival rate (%)				pH	
		1h	2h	3h	4h	0h	4h
1	*Bacillus subtilis* PA1	88.74	88.21	87.99	86.55	2.5	2.6
2	*B. licheniformis* PA5	91.64	92.74	86.88	84.54	2.5	2.7
3	*B. subtilis* PA6	86.93	86.18	85.78	85.37	2.5	2.7
4	*B. badius* PB4	NG	NG	NG	NG	2.5	2.8

Table 5: *Simulated acid and bile tolerance of selected Bacillus strains isolated from 'iru'.*

Key: NG - No growth.

Technological aspect of *Bacillus* species

One of the most important sources of microbial enzymes is the *Bacillus* species, it is also an important criterion in the selection of probiotics because digestive enzymes are natural substances utilized by the body to breakdown food and digest it and probiotics also improve digestion and restore normal intestinal flora; for an organism to be a probiotic it must be able to produce digestive enzymes [30]. Hence, all strains were tested for their abilities to produce enzymes like amylase, lipase, cellulase, and protease. Each isolate was able to produce at least one of the enzymes while only two isolates (*Bacillus subtilis* PA1 and *Bacillus alvei* PB5) were able to produce all four enzymes and *Bacillus pumilus* PB6 was not able to synthesize any enzyme. Olanbiwoninu and Fasiku [31] had earlier reported ability of *Bacillus subtilis* in the production of amylase and cellulase while Fasiku., *et al*. [32] isolated *Bacillus* spp. with potential of producing amylase, cellulase and protease. In this study, the results showed that not all same species of *Bacillus* can synthesize the same types of enzymes. This suggests that enzyme synthesis is a strain-specific characteristic. The cellulolytic activity ranged from 0.293 U/mL to 0.898 U/mL with *Bacillus licheniformis* PA8 having the highest activity. Highest amylolytic activity (0.0975 U/ml) was recorded by *Bacillus subtilis* PB10 while proteolytic activity ranged from 0.012 U/mL (*Bacillus subtilis* PA1) to 0.077 U/mL (*Bacillus subtilis* PB5). *Bacillus subtilis* PB5, PB6 and *Bacillus licheniformis* PA5, PB7 had highest lipase activity (0.6 U/mL) (Figure 2).

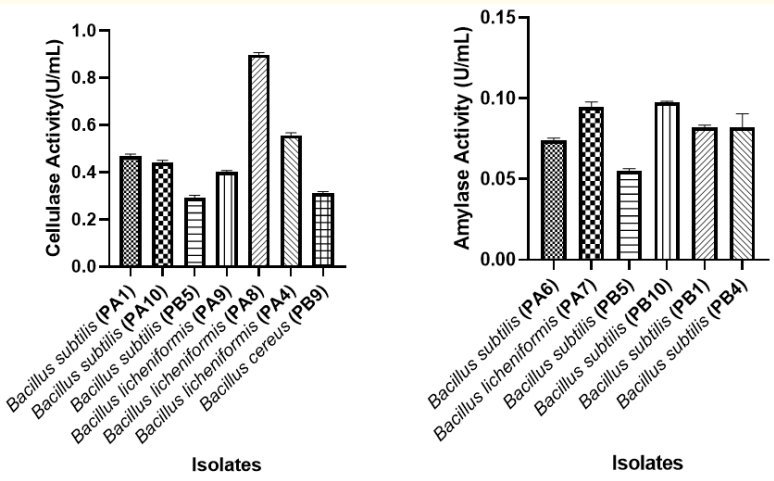

Figure 2a: *Cellulolytic activity of isolates.* ***Figure 2b:*** *Amylolytic activity of isolates.*

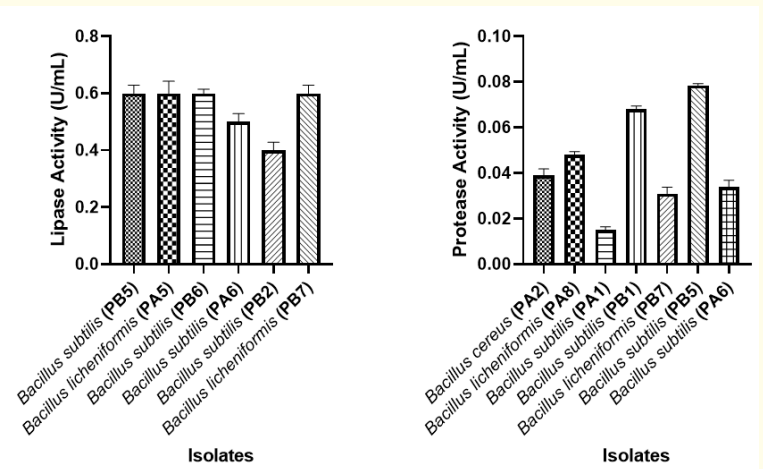

Figure 2c: *Lipolytic activity of isolates.* ***Figure 2d:*** *Proteolytic activity of isolates.*

Conclusion

Three potential probiotics (*Bacillus subtilis* PA1, PA6, and *Bacillus licheniformis* PA5) were successfully isolated from African fermented food origin, *iru*, with *Bacillus subtilis*, PA1 having the highest potential. *Bacillus subtilis* PA1, PA6, and *Bacillus licheniformis* PA5 were susceptible to many antibiotics, exhibited antimicrobial activity against *Listeria monocytogenes*, exhibited beta haemolysis, did not produce biofilm, demonstrated a high tolerance under simulated gastrointestinal conditions and were able to synthesize enzymes (cellulase, amylase, lipase and protease), which indicates their excellent potential as probiotics.

Conflict of Interest

The authors declare no conflict of interest.

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