British Biotechnology Journal
10(4): 1-12, 2016, Article no.BBJ.17698
ISSN: 2231–2927, NLM ID: 101616695

SCIENCEDOMAIN *international*
www.sciencedomain.org

Probiotic Properties and Antibiotic Resistance Pattern of *Bacillus* spp. Isolated from Two Types of Fermented Locust Bean (*iru*)

O. M. David[1*], J. L. Olagunju[1], A. A. Adebayo[1], T. T. Oluwaniyi[1] and M. O. Olajide[2]

[1]Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria.
[2]Department of Food Science, Joseph Ayo Babalola University, Ikeji-Arakeji, Osun State, Nigeria.

Authors' contributions

This work was carried out in collaboration between five authors. Author OMD designed the study while the four first authors developed the protocol and carried out the experimental. Authors OMD and AAA managed the analyses of the study. All the authors wrote the first draft of the manuscript and also read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2016/17698
Editor(s):
(1) Mahalingam Govindaraj, ICRISAT, Patancheru, India.
Reviewers:
(1) Nilesh Prakash Nirmal, The University of Queensland, Australia.
(2) Marcela Bianchessi da Cunha Santino, Universidade Federal de Sao Carlos, Brazil.
(3) Chen-Chin Chang, University of Kang Ning, Taiwan.
Complete Peer review History: http://sciencedomain.org/review-history/12243

Original Research Article

Received 24th March 2015
Accepted 8th May 2015
Published 10th November 2015

ABSTRACT

Bacillus spp. associated with two types of fermented African locust beans *iru woro* and *iru pete* were isolated and screened for probiotic potentials using standard microbiological techniques. The total bacterial counts for *iru woro* (pH 8.4) and *iru pete* (with pH 8.1) were 6.4314 and 6.4771 $\log_{10}$CFU/g respectively. In the two samples, the load of aerobic sporeformers were 6.2068 and 6.2553 $\log_{10}$CFU/g. In the samples *Bacillus subtilis* had the highest occurrence (44%), followed by *B. lichenliformis* (28%) and *B. megaterium* (24%) while *B. coagulans* had the least (4%). Only 28% of *Bacillus* isolates produced caseinase, while 28% produced haemolysin. Majority of these isolates showed tolerance to salt at concentrations less than 5% and also grew fairly at pH tending to neutral. *Bacillus subtilis* P14, *Bacillus lichenliformis* P12 and *Bacillus megaterium* P6 grew at 3.0% bile. Percentage hydophobicity, auto-aggregation and co-aggregation of the isolates ranged from -49.00 to 65.00%, -53.00 to 84.00% and -69.44 to 36.08% respectively. High level of antibiotic

**Corresponding author: E-mail: davidoluwole5@gmail.com;*

resistance (especially to first line antibiotics) was recorded among isolates. Most of the *Bacillus* species isolated from the *iru* samples had very poor probiotic properties. Molecular and *in vitro* probiotic properties of promising candidates are still open to investigation.

Keywords: Iru; probiotic; Bacillus spp.; locust beans; fermentation; biofilm.

1. INTRODUCTION

Fermentation is a process in which solid substrates are degraded by single or multiple cultures of microorganisms under a controlled environment to enhance high quality products. Fermentation process could either be submerged or solid state [1]. The fermentation of *iru* is by the solid state fermentation: A process characterized by complete or almost complete absence of free water. The water needed by the fermenting organisms is absorbed from the solid substrate matrix [2]. Fermentation of African locust bean is still by chance inoculation and constitute a vital body of indigenous knowledge used for food preservation which are acquired by observations and experiences, and passed on from generation to generation [3,4]. Though still at household level basis, chance or natural inoculum, unregulated conditions, sensory fluctuations, poor durability and unattractive packaging of *iru* still enjoy a wide acceptability in Nigeria [5].

Iru is an alkaline locust beans seeds fermented condiment consumed in Nigeria and other West Africa countries which could be eating immediately after fermentation as a snack unlike other African fermented condiments. *Iru* serves as a cheap source of protein and enhances the meatiness in soups, sauces and other dishes due to its high protein content [6]. There are two types of the fermented products; *iru pete* and *iru woro*. *Iru pete* and *iru woro* are the pasty and harder forms of *iru* respectively. Apart from the addition of softener during the second stage of boiling, longer period of fermentation produces soft and marshy paste of the cotyledons (*iru pete*). *Iru woro* is fermented for a shorter period of time to produce loose and whole cotyledons.

The bacteria isolated from fermented foods have been documented as to enhancing immunity, producing immune-stimulant and displaying probiotic properties such as, hypolipidemic, hepatoproctective and antibacterial; and had been found to be effective in treating gastroenteritis in man and animals [7]. Probiotics are harmless bacteria that promote the well-being of a host animal and contribute to the direct and/or indirect protection of the host animals against harmful bacteria.

Some species of *Bacillus* have been rated as generally regarded as safe (GRAS) and they are used to facilitate fast re-establishment of normal microbiota of the gastrointestinal tracts and prevent invasion and colonization of enteric pathogens and also lower cholesterol. *Bacillus* spp. has been reported to possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immunostimulation [8]. *Bacillus* species though aerobic organism have been reported to survive in the gut, withstand harsh condition and act beneficial role(s) to the animal [9,10]. *Bacillus* species has been reported to be the dominant bacterial species during fermentation of iru. The predominance of the species is due to the ability of its spores to withstand the second stage of boiling which lasts for minimum of 2 hours [11].

Bacillus species have not only been reported to possess probiotic properties but also stimulate the immunity of the animals [9,12]. *Bacillus* species effect the re-establishment of normal gastrointestinal tract microbiota and prevent its colonization of pathogenic strains of *Candida albicans* also Aderiye and David [12] reported the hypocholesterolemic activity of *Bacillus*. Despite the popular nutritional values of *iru* there is dearth of scientific information on the probiotic potentials of the *Bacillus* species associated with its fermentation which is the aim of this study.

2. MATERIALS AND METHODS

2.1 Target Sample and Description

Two varieties of freshly fermented locust bean condiments; *iru woro* and *iru pete* (Fig. 1) were purchased from Oja-Oba in Ibadan, Oyo State, Nigeria, and were preserved at 4℃ before the laboratory analysis.

2.2 Determination of the pH of *iru* Samples

Direct measurement was employed to determine the pH of *iru* samples. Five gram of *iru* sample was emulsified in 45 mL of distilled water following a vortex mixing. The pH was directly measured using pocket sized pH meter (Model H196107, Hanna Instruments).

2.3 Microbiological Analyses

2.3.1 Determination of total bacterial count

Iru samples were serially diluted and inoculated on sterile Nutrient agar (Oxoid) and incubated for 24 h at 37℃. The experiment was performed in triplicate and repeated twice. The colonies developed on the plates were counted after incubation using colony counter (Gallenkamp, England).

2.3.2 Isolation of *Bacillus* spp. from *iru* samples

The method of Barbosa et al. [8] with little modification was used for the isolation of *Bacillus* spp. from *iru* samples. Five grams (5 g) of ground *iru* samples were suspended in 10 mL distilled water in sterile bottles with vigorous shaking. The suspension was ten-fold diluted and heated to 65℃ for 45 min. The suspended *iru* samples was further diluted in absolute ethanol (1:1, v/v) and allowed to stand for 1 h at room temperature. 1 mL of the resultant solutions were inoculated on Hi-Chrome *Bacillus* Agar (HCBA) (HiMedia M1651, India) using pour plate method and incubated aerobically at 37℃ for 24 h. Discrete colonies were sub-cultured on HCBA and single colonies were transferred into the slant. The identity of the isolates was determined by Gram reactions, catalase, indole, Voges-Proskauer and Methyl-Red test, utilization of citrate, fermentation of carbohydrate (arabinose, fructose, galactose, inositol, mannitol, mannose, rhamnose, ribose, sorbose and xylose).

2.4 Determination of Probiotic Properties of *Bacillus* spp. Isolated from *iru* Samples

2.4.1 Detection of gelatinase production

Nutrient agar supplemented with 0.4% by weight, of gelatin (BDH), with a final pH 7.2 was prepared and the isolates were streaked on the plates and incubated for 48hours at 37℃. The cultures were observed for growth and subsequently flooded with 10 mL of Frazier solution (Mercuric chloride, 15.0 g in 20 mL of 37% v/v hydrochloric acid, made up to 100 mL by adding distilled water). The plates which showed area of opaque layer with zone of clearance around the colonies were taken as positive for gelatin hydrolysis according to [13].

2.4.2 Detection of haemolysin production

Brain heart infusion agar (Oxoid, UK) supplemented with 5% human blood was used for detection of haemolysin production by the isolates. The medium was inoculated with test isolates using streaking method and incubated at 37℃ for 24 h. Haemolytic activity was observed as β-haemolysis surrounding bacterial colonies in the plates.

2.4.3 Detection of caseinase production

Bacillus species were inoculated onto Trypticase Soy agar (TSA) (Oxoid, UK) supplemented with 1% skim milk (w/v) using streaking method and incubated at 37℃ for 24 h. Caseinase production was observed with zone of clearance around isolates according to [14].

2.4.4 pH tolerance test

Each of the isolates was inoculated into 5 ml of 0.1 M phosphate buffer solution with different pH (ranging from 3.0-12.0), adjusted with 1 M hydrochloric acid, and incubated for 3 h. Following a thorough shaking, 1mL of inoculum was streaked on molten nutrient agar and incubated 37℃ for 24 h after which the plates were observed for growth.

2.4.5 Salt and bile tolerance test

Isolates were streaked on nutrient agar plates containing sodium chloride (NaCl) and bovine bile separately and incubated at 37℃ for 24 h. Bacterial growth on the plates was observed by the presence of colonies or confluent of bacteria.

2.5 Qualitative and Semi-quantitative Detection of Biofilm Production

Biofilm formation among the isolates was detected by the method of [15]. The isolates were radially streaked on nutrient agar supplemented with Congo red dye. The plates were incubated for 24 h at 37℃. Isolates with black colonies on Congo red agar were taken for biofilm production. The quantity of biofilm formed by isolates was further determined by inoculating them into Mueller Hilton broth (MHB) (HiMedia, India) and incubated at 37℃ for 72 h; a sterile MHB was used as control. The broth was discarded and adherent bacterial cells were stained with 1% Crystal-violet (Merck, France) for 10 m. Excess stain was rinsed off and air dried. The dry tube was bleached with absolute ethanol and the optical density was measured at 520 nm

(OD_{520}) using spectrophotometer (WPA Linton Cambridge, UK). Quantity of biofilm formed was classified as strong ($OD_{520} \geq 0.30$) or week ($OD_{520} < 0.30$).

2.6 Hydrophobicity Assay

Hydrophobicity of the isolates was determined by hydrocarbon partitioning with little modification of the method of [16]. Bacterial cultures were grown in Mueller Hilton broth and incubated at 37℃ for 24 h. The bacterial suspensions were spun at 10,000xg for 10 m. The pellets were washed twice with 0.1 M phosphate buffer saline (pH 7.0) and optical densities of the suspensions were measured at OD_{600} and adjusted to OD_{600} =1.0. 2 mL of the bacterial suspensions were mixed with 0.5 mL of benzene (after 10 min of pre-incubation at room temperature), vortex-mixed for 2 m and left for 4h at room temperature. After phase separation, the optical density of the aqueous phase was measure at OD_{600} again. Hydrophobicity was calculated according to the equation;

$$\text{Hydrophobicity \%} = [(A_o - A_1)/ A_o] \times 100$$

Where A_o represented the initial OD and A_1 was the OD of aqueous phase

2.7 Auto-aggregation Assay

The method of Kos et al. [17] was modified to determine the auto-aggregation of isolated *Bacillus* spp. Bacterial cultures were grown in MHB at 37℃ for 24 h. The cell were washed twice with 1.0 M phosphate buffer saline (pH 7.0), and the OD was adjusted to 0.25±0.05 at 600 nm wavelength to obtain bacterial count approximately 10^8 CFU/mL. Five milliliters of cell suspensions were vortex-mixed for 30s and the optical density (OD_{600}) at 0 h was measured. After 5 h of incubation at room temperature, optical densities (OD_{600}) of the upper suspensions were determined. Auto-aggregation was calculated according to the equation;

$$1-(A_t / A_o) \times 100$$

Where A_o represented the OD at time (t) = 0 and A_t was the OD at t = 5 h

2.8 Co-aggregation Assay

Cells of test isolates *Bacillus* spp. and *Escherichia coli* were separately harvested and OD_{600} was adjusted to 0.25±0.05, following procedures earlier described. Equal volume (2 mL) of each of the test isolates were mixed with that of *E. coli*. 4 mL of each bacterial suspension was separately prepared as controls and incubated at room temperature for 4h. Optical density at 600 nm wavelength of the mixture and bacterial suspension alone were determined and co-aggregation was calculated according as:

$$\{[(A_A + A_B)/2 - A_{A+B}]/ (A_A + A_B)/2\} \times 100$$

Where A_A represented the OD_{600} of *Bacillus* sp. A_B was the OD_{600} of *E. coli* and A_{A+B} was the OD_{600} of the mixture of *Bacillus* sp. and *E. coli* after 4 h.

Iru pete *Iru woro*

Fig. 1. The two varieties of fermented locust beans, *iru*

2.9 Antibiotic Sensitivity Test

The isolates grown at 37℃ in Mueller-Hilton broth (Oxoid) for 18 h was diluted to an OD_{600} of 0.1 (0.5 McFarland Standard) and stored at 4℃. The disc diffusion method was used for susceptibility testing as described by Clinical and Laboratory Standard Institute (2012). The isolates were tested against eight commercial antibiotic disks (Abtek Biologicals Limited) with different concentrations which included: Ampicillin (25 µg), augmentin (30 µg), ceftazidine (30 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), nitrofurantoin (300 µg) and ofloxacin (5 µg). The diameters of the zone of inhibition were measured to the nearest whole millimeter and interpreted according to CLSI guideline [18].

2.10 Statistical Analyses

Statistical analysis was done using SPSS (version 17) to determine frequency distribution, analysis of variance (ANOVA), Duncan Multiple Range and Pearson correlation coefficient.

3. RESULTS

The bacterial load of the two samples of fermented locust beans is presented in Table 1. The total bacterial count of the two samples were 6.4314 and 6.4771 $\log_{10}$CFU/g to $1.68x10^6$ CFU/g in *iru woro* and *iru pete* respectively. *Iru pete* had higher *Bacillus* spp. count (6.2553 $\log_{10}$CFU/g) than *iru woro* (6.2068 $\log_{10}$CFU/g). Biochemical and morphological characterization of the *Bacillus* species isolated from the samples consisted of *Bacillus subtilis*, *Bacillus lichenliformis, Bacillus megaterium* and *Bacillus coagulans* (Table 2).

Twenty two (88%) of the *Bacillus* spp. produced haemolysin while only 28% produced caseinase. The rate of gelatinase production was pronounced among the *B. subtilis* and *B. licheliformis* as shown in Table 3*.* Also represented in Table 4 is level of tolerance of the isolates to different concentrations of NaCl.

As presented in Table 5, most of the isolates were able to grow at pH level tending to 7 but the growth was better as the pH increased toward alkalinity. Only three of the isolates; *B. subtilis* P1, P5 and *B. lichenliformis* P8, were able to survive at an acidic condition. *Bacillus lichenliformis* P8 was able to grow at all the levels of pH value tested.

The isolates were screen for their ability to grow in the presence of bile (Table 6). Majority of the isolates showed a significant growth at lower concentration of bile. Only *B. subtilis* P14, *B. lichenliformis* P12 and *B. megaterium* P6 were able to grow when the volume of bile was increased to 4.0 % w/v of bile.

The ability of the isolates to produce biofilm was assayed for, qualitatively (and quantitatively for producers). Only 28% of the total isolates produced biofilm. All the isolates that produce biofilm were strong biofilm formers (Table 7). As shown in Table 8, *B. subtilis* P5 showed the highest hydrophobicity of 65%, followed by *B. megaterium* P15 and *B. megaterium* P7 with 49% and 47% respectively. *Bacillus lichenliformis* P12 showed the highest auto-aggregation. The hydrophobicity of the auto-aggregation of the isolates from *iru pete* were higher than the isolates from *iru woro.* On the other hand, the co-aggregation value of the isolates from *iru woro* was higher than those from *iru pete.* There is no significant difference in the hydrophobicity and the cell aggregations of the isolates from both samples.

The result of the sensitivity test of *Bacillus* spp. isolated from *iru* samples to eight common antibiotics is shown in Table 9. The isolates showed varying resistance to antibiotics used. All the species had very low resistance to ofloxacin and ciprofloxacin. Hence, ofloxacin and ciprofloxacin inhibited the growth of the isolates. Only *B. coagulans* was totally resistant to gentamicin (GEN) and nitrofurantoin (NIT). The isolates were not susceptible to ceftazidime (CAZ), cefuroxime (CRX), ampicillin (AMP) and augmentin (AUG).

Table 1. Spore-forming bacteria count ($\log_{10}$CFU/g) in *iru* samples

Bacterial count	*Iru* samples	
	Iru woro	***Iru pete***
Total bacterial count (CFU/g)	6.4314±1.3423	6.4771±2.4162
Spore-former count (CFU/g)	6.2068±2.814	6.2553±1.1132
pH	8.4	8.1

Table 2. Percentage distribution of *Bacillus* spp. in *iru* samples

Isolates	Occurrence no (%)	Distribution by samples	
		Iru woro	***Iru pete***
Bacillus subtilis	11(44)	4	7
Bacillus lichenliformis	7(28)	4	3
Bacillus megaterium	6(24)	1	5
Bacillus coagulans	1(4)	ND	1
Total	25		

Key: ND - Not detected

Table 3. Enzyme production among *Bacillus* spp. isolated from *iru* samples

Isolates	Enzymes		
	Haemolysin	**Caseinase**	**Gelatinase**
Bacillus subtilis P1	+	+	+++
Bacillus subtilis P2	+	-	-
Bacillus subtilis P4	+	-	++
Bacillus subtilis P5	+	-	++
Bacillus subtilis P11	+	-	++
Bacillus subtilis P13	+	-	+
Bacillus subtilis P14	+	+	++
Bacillus coagulans P10	+	-	+
Bacillus lichenliformis P8	+	-	+
Bacillus lichenliformis P9	+	-	++
Bacillus lichenliformis P12	+	+	+++
Bacillus megaterium P3	+	-	-
Bacillus megaterium P6	+	+	++
Bacillus megaterium P7	+	-	+
Bacillus megaterium P15	+	-	+
Bacillus megaterium P16	-	-	+
Bacillus subtilis W3	+	-	-
Bacillus subtilis W4	+	-	+
Bacillus subtilis W5	+	-	+
Bacillus subtilis W7	+	-	-
Bacillus lichenliformis W1	+	-	+
Bacillus lichenliformis W2	+	-	++
Bacillus lichenliformis W9	-	+	++
Bacillus lichenliformis W10	+	+	-
Bacillus megaterium W6	-	+	+

Production (+), Moderate Production (++), Strong Production (+++) and No Production (-)

4. DISCUSSION

The present study explores the probiotic properties of *Bacillus* spp. isolated from two samples of fermented African locust bean: *iru woro* and *iru pete*. The density of bacteria recorded for the samples were considered very high, considering their usage as additive in food. This may be as a result of fermentation, involving interactions between different microorganisms [19], coupled with the mode of preparation, handling and packaging [20]. The difference in the microbial loads of the two products may be due to the addition of the cotyledon softner and the length of fermentation. Due to their ability to form spores and withstand a range of variable environmental conditions, *Bacillus* spp. has been reported to adapt easily to diverse habitats [14]; this is supported by the result of this study. A total of twenty-five species of *Bacillus* was selectively isolated using Hi Chrome Bacillus Agar, in line with previous studies of Vinod and More [21] followed by biochemical characterization.

Bacillus spp. has been implicated in the fermentation of African locust bean [22-25]. However, there is a paucity of information about their probiotic potentials, despite the fact that most of the microorganisms associated with other fermented foods have long been documented as to enhancing immunity, producing immunostimulants and displaying probiotic properties [6,8,26-28].

Table 4. Salt tolerance of *Bacillus* spp. isolated from *iru* samples

Isolates	Salt concentration (w/v %)							
	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1
Bacillus subtilis P1	-	-	-	-	-	+	+	+
Bacillus subtilis P2	-	-	-	-	+	+	+	+
Bacillus subtilis P4	-	-	-	-	+	-	+	+
Bacillus subtilis P5	-	-	-	-	-	-	+	+
Bacillus subtilis P11	-	-	-	-	-	-	+	+
Bacillus subtilis P13	-	-	-	+	+	+	+	+
Bacillus subtilis P14	-	-	-	-	+	+	+	+
Bacillus coagulans P10	-	-	-	-	-	+	+	+
Bacillus lichenliformis P8	-	-	-	-	+	+	+	+
Bacillus lichenliformis P9	-	-	-	-	-	-	+	+
Bacillus lichenliformis P12	-	-	-	-	+	+	+	+
Bacillus megaterium P3	-	-	-	-	+	+	+	+
Bacillus megaterium P6	-	-	-	+	+	+	+	+
Bacillus megaterium P7	-	-	-	-	-	-	+	+
Bacillus megaterium P15	-	-	-	-	+	+	+	+
Bacillus megaterium P16	-	-	-	-	+	+	+	+
Bacillus subtilis W3	-	-	-	-	-	-	+	+
Bacillus subtilis W4	-	-	-	-	-	-	+	+
Bacillus subtilis W5	-	-	-	-	+	+	+	+
Bacillus subtilis W7	-	-	-	-	-	-	+	+
Bacillus lichenliformis W1	-	-	-	-	-	+	+	+
Bacillus lichenliformis W2	-	-	-	-	-	-	+	+
Bacillus lichenliformis W9	-	-	-	-	-	+	+	+
Bacillus lichenliformis W10	-	-	-	-	+	+	+	+
Bacillus megaterium W6	-	-	-	-	+	+	+	+

Key: Growth (+), No Growth (-)

Table 5. pH Tolerance of *Bacillus* spp. isolated from *iru* samples

Isolates	pH Level									
	3	4	5	6	7	8	9	10	11	12
Bacillus subtilis P1	+	+	+	+	-	-	+	+	+	+
Bacillus subtilis P2	-	-	+	-	+	+	+	+	+	+
Bacillus subtilis P4	-	+	+	-	-	+	+	+	+	+
Bacillus subtilis P5	+	-	-	+	+	+	+	+	+	+
Bacillus subtilis P11	-	-	-	+	+	+	+	+	+	+
Bacillus subtilis P13	-	-	-	+	+	+	+	+	+	+
Bacillus subtilis P14	-	-	-	+	+	+	+	+	+	+
Bacillus coagulans P10	-	-	+	+	+	+	+	+	+	+
Bacillus lichenliformis P8	+	+	+	+	+	+	+	+	+	+
Bacillus lichenliformis P9	-	+	-	+	-	+	+	+	+	+
Bacillus lichenliformis P12	-	-	+	+	+	+	+	+	+	+
Bacillus megaterium P3	-	-	+	+	+	+	+	+	+	+
Bacillus megaterium P6	-	-	+	+	+	+	+	+	+	+
Bacillus megaterium P7	-	-	-	+	-	+	+	+	+	+
Bacillus megaterium P15	-	-	-	-	-	+	+	+	+	+
Bacillus megaterium P16	-	-	-	-	+	+	+	+	+	+
Bacillus subtilis W3	-	-	+	+	+	+	+	+	+	+
Bacillus subtilis W4	-	-	+	+	-	+	+	+	+	+
Bacillus subtilis W5	-	-	+	+	+	+	+	+	+	+
Bacillus subtilis W7	-	-	-	+	+	+	+	+	+	+
Bacillus lichenliformis W1	-	-	-	+	+	+	+	+	+	+
Bacillus lichenliformis W2	-	-	+	+	-	+	+	+	+	+
Bacillus lichenliformis W9	-	-	+	+	-	+	+	+	+	+
Bacillus lichenliformis W10	-	-	-	+	-	+	+	+	+	+
Bacillus megaterium W6	-	-	-	-	-	+	+	+	+	+

Key: Growth (+), No Growth (-)

Table 6. Bile tolerance of *Bacillus* spp. isolated from *iru* samples

Isolates	Bile concentration (% w/v)												
	7.0	6.5	6.0	5.5	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0
Bacillus subtilis P1	-	-	-	-	-	-	-	-	+	+	+	+	+
Bacillus subtilis P2	-	-	-	-	-	-	-	-	+	+	+	+	+
Bacillus subtilis P4	-	-	-	-	-	-	-	+	+	+	+	+	+
Bacillus subtilis P5	-	-	-	-	-	-	-	-	+	+	+	+	+
Bacillus subtilis P11	-	-	-	-	-	-	-	+	+	+	+	+	+
Bacillus subtilis P13	-	-	-	-	-	-	-	-	+	+	+	+	+
Bacillus subtilis P14	-	-	-	-	-	-	+	+	+	+	+	+	+
Bacillus coagulans P10	-	-	-	-	-	-	-	-	-	-	+	+	+
Bacillus lichenliformis P8	-	-	-	-	-	-	-	+	-	+	+	+	+
Bacillus lichenliformis P9	-	-	-	-	-	-	-	-	-	+	+	+	+
Bacillus lichenliformis P12	-	-	-	-	-	-	+	+	+	+	+	+	+
Bacillus megaterium P3	-	-	-	-	-	-	-	-	-	+	+	+	+
Bacillus megaterium P6	-	-	-	-	-	-	+	+	+	+	+	+	+
Bacillus megaterium P7	-	-	-	-	-	-	-	+	+	+	+	+	+
Bacillus megaterium P15	-	-	-	-	-	-	-	+	+	+	+	+	+
Bacillus megaterium P16	-	-	-	-	-	-	-	-	-	-	+	+	+
Bacillus subtilis W3	-	-	-	-	-	-	-	-	-	+	+	+	+
Bacillus subtilis W4	-	-	-	-	-	-	-	-	-	-	+	+	+
Bacillus subtilis W5	-	-	-	-	-	-	-	+	+	+	+	+	+
Bacillus subtilis W7	-	-	-	-	-	-	-	-	+	+	+	+	+
Bacillus lichenliformis W1	-	-	-	-	-	-	-	-	-	-	+	+	+
Bacillus lichenliformis W2	-	-	-	-	-	-	-	-	+	-	+	+	+
Bacillus lichenliformis W9	-	-	-	-	-	-	-	-	+	+	+	+	+
Bacillus lichenliformis W10	-	-	-	-	-	-	-	+	+	+	+	+	+
Bacillus megaterium W6	-	-	-	-	-	-	-	-	+	+	+	+	+

Key: Growth (+), No Growth (-)

Table 7. Biofilm formation of *Bacillus* spp. isolated from *iru* samples

Isolates	Biofilm formation		Type of biofilm
	Qualitative assay	Optical density reading (OD_{520})	
Bacillus subtilis P1	+	0.50	Strong
Bacillus subtilis P2	-	ND	-
Bacillus subtilis P4	-	ND	-
Bacillus subtilis P5	-	ND	-
Bacillus subtilis P11	+	0.37	Strong
Bacillus subtilis P13	-	ND	-
Bacillus subtilis P14	+	0.45	Strong
Bacillus coagulans P10	-	ND	-
Bacillus lichenliformis P8	-	ND	-
Bacillus lichenliformis P9	-	ND	-
Bacillus lichenliformis P12	+	0.43	Strong
Bacillus megaterium P3	-	ND	-
Bacillus megaterium P6	+	0.47	Strong
Bacillus megaterium P7	+	0.56	Strong
Bacillus megaterium P15	-	ND	-
Bacillus megaterium P16	-	ND	-
Bacillus subtilis W3	-	ND	-
Bacillus subtilis W4	-	ND	-
Bacillus subtilis W5	-	ND	-
Bacillus subtilis W7	-	ND	-
Bacillus lichenliformis W1	-	ND	-
Bacillus lichenliformis W2	-	ND	-
Bacillus lichenliformis W9	-	ND	-

Isolates	Biofilm formation		Type of biofilm
	Qualitative assay	Optical density reading (OD_{520})	
Bacillus lichenliformis W10	+	0.40	Strong
Bacillus megaterium W6	+	0.52	Strong

Key: Biofilm formed (+), No Biofilm formed (-), ND = Not determined

Table 8. Hydrophobicity and aggregation status of *Bacillus* spp. isolated from *iru* samples

Isolates	Hydrophobicity (%)	Cell aggregation (%)	
		Auto-aggregation	Co-aggregation
Bacillus subtilis P1	-6.00	-17.00	-2.13
Bacillus subtilis P2	15.00	-53.00	-33.33
Bacillus subtilis P4	4.00	5.00	-5.88
Bacillus subtilis P5	65.00	-7.00	32.00
Bacillus subtilis P11	34.00	27.00	-15.46
Bacillus subtilis P13	0.00	19.00	13.98
Bacillus subtilis P14	28.00	29.00	35.14
Bacillus coagulans P10	-23.00	-9.00	9.28
Bacillus lichenliformis P8	-29.00	35.00	21.84
Bacillus lichenliformis P9	26.00	7.00	1.59
Bacillus lichenliformis P12	24.00	84.00	27.62
Bacillus megaterium P3	-49.00	28.00	20.33
Bacillus megaterium P6	6.00	20.00	16.54
Bacillus megaterium P7	47.00	6.00	26.92
Bacillus megaterium P15	49.00	15.00	-69.44
Bacillus megaterium P16	11.00	4.00	24.23
Bacillus subtilis W3	18.00	6.00	-16.33
Bacillus subtilis W4	-2.00	37.00	21.50
Bacillus subtilis W5	9.00	-26.00	-3.70
Bacillus subtilis W7	-29.00	10.00	36.08
Bacillus lichenliformis W1	37.00	-16.00	-22.86
Bacillus lichenliformis W2	-10.00	4.00	11.83
Bacillus lichenliformis W9	-5.00	2.00	8.89
Bacillus lichenliformis W10	-3.00	-10.00	2.50
Bacillus megaterium W6	-49.00	26.00	30.69

Probiotics as more recently described as live microorganisms serving beneficial effects to humans and animals when consumed in adequate amounts [29,30]. *Bacillus clausii, Bacillus subtilis, Bacillus pumillus, Bacillus coagulans* and *Bacillus cereus* were reported to possess probiotic properties by Riddell et al. [31] and Aderiye and David [12]. Mach [32] stated that lack of probiotic bacteria in the gut flora is the main cause of many diseases of today. Essential qualities looked out for in any microorganism that makes it exploitable as probiotic include; safety, viability during processing and storage, antagonistic effect against pathogens, capable of surviving in the intestinal ecosystem [33,34].

The present study has revolved its evaluation of potential probiotics around all these aforementioned essentials, hence demonstrating attributes of isolated bacilli from *iru* samples including production of enzymes; bile, salt and pH tolerance, biofilm formation, cellular aggregation and cell surface hydrophobicity. Apparently, the present study revealed that majority of the screened *Bacillus* spp. could not withstand the presence of bile at higher concentrations; except for *B. subtilis* P14, *B. cereus* P12 and *B. megaterium* P6 that were able to grow at bile concentration of 4%. Deshpande et al. [35] reported similar case of a potential probiotics (*Lactobacillus* spp.) that was able to withstand 0.5% of bile salt.

The enzyme activities in this study revealed that majority of tested *Bacillus* spp. produced haemolysin and gelatinase better, while few of them possess the ability to produce caseinase. Similar study reported that *Bacillus* spp. produced caseinase [14]. Previous studies have

Table 9. Percentage antibiotic resistance of *Bacillus* spp. isolated from *iru* samples

Isolates	Antibiotics							
	CAZ	CRX	GEN	CPR	OFL	AUG	NIT	AMP
Bacillus subtilis (n=11)	100	100	64	18	18	100	55	100
Bacillus lichenliformis (n=7)	100	100	57	29	29	100	57	100
Bacillus megaterium (n=6)	100	83	50	0	0	83	33	83
Bacillus coagulans (n=1)	100	100	100	0	0	100	100	100

Key: CAZ- Ceftazidine, CRX- cefuroxime, GEN- Gentamycin, CPR- Ciprofloxacin, OFL- Ofloxacin, AUG- Augmentin, NIT- Nitrofurantoin, AMP- Ampicillin

however established the fact that *Bacillus* spp. secretes many exoenzymes; which are very efficient in breaking down large molecular substances into smaller units [36].

Vesterlund et al. [37], Abderrahmen et al. [14] and Anwar et al. [16] described cell surface hydrophobicity of a potential probiotics as one of most important factors which govern the mechanism of bacterial adhesion to inanimate and biological surfaces. The cell surface protein (S-layer protein) may have contributed to variation in the aggregation abilities of the species [38,39]. Saidi et al. [40] have long reported that aggregation ability is related to cell adherence properties as the ability of the bacteria to form biofilm could be qualitatively determined using Congo red assay [14,41].

The result of this study demonstrated a high level of antibiotic resistance among the tested isolates and this is similar to the earlier report of Abderrahmen et al. [14], Ravi et al. [42] and Dai et al. [43].

The present study has established that most of the *Bacillus* spp. isolated from *iru* could not be good probiotic agents. Some of the isolates have pathogenic factor(s) and are resistant to most antibiotics tested against them. *Bacillus subtilis* P14, *Bacillus lichenliformis* P12 and *Bacillus megaterium* P6 could be considered as probiotic candidate however, the molecular characterization including plasmid profiling of these isolates are recommended.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ajayi OA. Bacteriology and qualitative study of African locust bean (*Parkia biglobosa*). Open Journal of Social Sciences. 2014;2:73-78.
2. Adebiyi OA. Fungal degradation of cowpea seed hull for utilization by meatytype (broiler) chicken. Ph.D Thesis, University of Ibadan, Ibadan; 2007.
3. Aworh OC. The role of traditional food processing technologies in National Development: The West African experience. International Union of Food Science and Technology. 2008;4(2):1-18.
4. Abdel AA, Dardir HA. Hygienic quality of local traditional fermented skimmed milk (Laban Rayb) sold in Egypt. World Journal of Dairy and Food Sciences. 2009; 4(2):205-209.
5. Olanrewaju OO, Victor OO, Titilayo TA. Safety of small-scale food fermentations in developing countries. Internet Journal of Food Safety. 2009;11:29-34.
6. Campbell-Platt G. Traditional West African Food. In Ethnic Food Symposium, Twentieth Annual Conference of The Institution of Food Science and Technical Technology IKST Proceedings. 1980;17: 214-218.
7. Aderiye BI, Laleye SA, Odeyemi AT. Hypolipidemic effect of *Lactobacillus* and *Streptococcus* species from some Nigerian fermented foods. Research Journal of Microbiology. 2007;2(6):538-544.
8. Barbosa TM, Serra CR, La Ragione RM, Woodward MJ, Henriques AO. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Applied Environmental Microbiology. 2005;71:968-978.
9. Felske ADM. Ecology of *Bacillus* species in soil. In: Ricca E, Henriques AO, Cutting SM. (ed), Bacterial spore formers: Probiotics and emerging applications. Horizon Scientific Press, London, United Kingdom. 2004;35-44.
10. Tam NK, Uyen NQ, Hong HA, Duc LH, Hoa TT, Serra CR, Henriques AO, Cutting SM. The intestinal life cycle of *Bacillus subtilis* and close relatives. Journal of Bacteriology. 2006;188:2692–2700.

11. Ogbadu LJ, Okagbue RN. Fermentation of African locust bean (*Parkia biglobosa*) Seeds: Involvement of different species of *Bacillus*. Food Microbiology. 1988;5:195-199.
12. Aderiye BI, David, OM. *In vivo* evaluation of hypolipidemic potentials of *Bacillus* species isolated from fermented locust bean (*Pakia fillicoides* Welw) seeds (Iru). British Microbiology Research Journal. 2013;3(4):574-584.
13. Su YA, Sulavik MC, He P, Makinen KK, Makinen PL, Fiedler S, Wirth R, Clewell DB. Nucleotide sequence of the gelatinase gene (gelE) from *Enterococcus faecalis* subs. liquefaciens. Infection and Immunity. 1991;59:415–420.
14. Abderrahmen M, Nadia L, Abir B, Amina B. Evaluation of the probiotic properties of *Bacillus* spp. strains isolated from Tunisian hypersaline environments. African Journal of Microbiology Research. 2014;8(4):398-405.
15. Chaleb K, Chehab O, Zmantar T, Rouabhia M, Mahdouani K, Bakhrouf A. In vitro effect of pH and ethanol on biofilm formation by clinical positive *Staphylococcus epidermidis* strains. Annals of Microbiology. 2007;57:431-437.
16. Anwar A, Abdulla A, Thikra, AA, Saeed AM. Adhesion, autoaggregation and hydrophobicity of Six *Lactobacillus* Strains. British Microbiology Research Journal. 2014;4(4):381-391.
17. Kos B, Suskovic J, Vukovic S, Simpraga M, Frece J, Matosic S. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. Journal of Applied Microbiology. 2003;94:981-987.
18. CLSI. Performance standards for antimicrobial susceptibility testing; fifteenth informational supplement, Clinical and Laboratory Standard Institute Wayne, Pa. M100- S15,25:1.
19. Omafuvbe BO, Abiose SH, Shonukan OO. Fermentation of soybean (*Glycine max*) for soy-daddawa production by starter cultures of Bacillus. Food Microbiology. 2003;19: 561-566.
20. Osho A, Mabekoje OO, Bello OO. Comparative study on the microbial load of gari, elubo-isu and *iru* in Nigeria. African Journal of Food Science. 2010;4(10):646-649.
21. Vinod AS, More SM. Isolation, identification and characterization of *Bacillus* species from Lonar Lake for production of cyclodextrin glycosyl-transferase. International Journal of Current Microbiology and Applied Science. 2013;2(12):14-23.
22. Odunfa SA, Ready NR, Peirson MD, Salunkhe DK. Dawadawa. *In* Legume-Based Fermented Foods. CRS Press, Boca Raton, Florida. 173-189.
23. Adenike AOO, Mopelola OO, Ayansina ADV. Microbial studies and biochemical characteristics of controlled fermented Afiyo- a Nigerian fermented food condiment from *Prosopis africana.* Pakistan Journal of Nutrition. 2007;6(6): 620-627.
24. Adeleke RO, Abiodun OA. Physico-chemical properties of commercial local beverages in Osun State, Nigeria. Pakistan Journal of Nutrition. 2010;9(9): 853-855.
25. Falegan CR. Microbiology profile and biochemical characteristics of commercial 'ogiri' samples from South Western Nigeria. Journal of Microbiology and Food Sciences. 2011;1(2):187-203.
26. Sanders ME, Morelli L, Tompkins TA. Spore formers as human probiotics: *Bacillus, Sporolactobacillus* and *Brevibacillus*. Comprehensive Reviews in Food Science and Food Safety. 2003;2: 101-110.
27. Selvan R, Maheswari P, Kavitha P, Ravichandran M, Sas B, Ramchand CN. Effects of *Bacillus subtilis* PB6, a natural probiotic on colon mucosal inflammation and plasma cytokine level inflammatory bowel diseases. Indian Journal of Biochemistry and Biophysics. 2005;46:79-85.
28. Cartman ST, La Ragione RM, Woodward MJ. *Bacillus subtilis* spores 248 germinate in the chicken gastrointestinal tract. Applied Environmental Microbiology. 2008; 249(74):5254–5258.
29. Guarner F, Schaafsma GJ. Probiotics. International Journal of Food Microbiology. 1998;39:237-238.
30. Rijkers GT, De Vos WM, Brummer RJ, Morelli L, Corthier G, Marteau P. Health benefits and health claims of probiotics: Bridging science and marketing. British Journal of Nutrition. 2011;106(9):1291-1296.
31. Riddell JB, Gallegos AJ, Harmon L, McLeod KR. Addition of a *Bacillus* based probiotic to the diet of pre ruminant calves:

Influence on growth, health and blood parameters. International Journal of Applied Research in Veterinary Medicine. 2010;8:78-84.

32. Mach T. Clinical usefulness of probiotics against chronic inflammatory bowel diseases. Journal of Physiology and Pharmacology. 2006;57(9):23–33.
33. Lin W, Hwang C, Chen L, Tsen H. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Microbiology. 2006;23:74-81.
34. Alander M, Korpela R, Saxeline M, Vilpponen-Salmela T, Matilla-Sandholm T, Wright A. Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. Letters in Applied Microbiology. 1997;24:361-364.
35. Deshpande KG, Dolas CB, Chavan NS. Investigation of tolerance of *Lactobacillus casei* to the presence of acids, bile salts and deconjugation of bile salts. International Journal of current Microbiology and Appied Science. 2014; 3(7):600-612.
36. Ninawe AS, Selvin J. Probiotics in shrimp aquaculture: Avenues and challenges. Critical Reviews in Microbiology. 2009; 35:43-66.
37. Vesterlund S, Paltta J, Karp M, Ouwehand AC. Adhesion of bacteria to resected human colonic tissue: Quantitative analysis of bacterial adhesion and viability. Research in Microbiology. 2005;156:238-244.
38. Goh YJ, Klaenhammer TR. Functional roles of aggregation- promoting- like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. Applied and Environmental Microbiology. 2010;76: 5005-5015.
39. Del Re B, Sgorbati B, Miglioli M, Palenzona D. Adhesion autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology. 2000;31:438-442.
40. Saidi N, Snoussi M, Usai D, Zanetti S, Bakhrouf A. Adhesive properties of *Aeromonas hydrophila* strains isolated from Tunisian aquatic biotopes. African Journal of Microbiology Research. 2011; 31:5644-5655.
41. FAO/WHO. Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Expert Consultation Group: Cordoba, Argentina; 2001.
42. Ravi AV, Musthafa KS, Jegathammbal G, Kathiresan K, Pandian SK. Screening and evaluation of probiotics as a biocontrol agent against pathogenic vibrios in marine aquaculture. Letters in Applied Microbiology. 2007;45:219-223.
43. Dai L, Wu CM, Wang MG, Wang Y, Wang Y, Huang SY. First report of the multi-drug resistance gene cfr and the phenicol resistance gene fex Aina *Bacillus* strain from swine feces. Antimicrobial Agents and Chemotheraphy. 2010;54:169-179.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/12243