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In vivo Evaluation of Hypolipidemic Potentials of *Bacillus* Species Isolated from Fermented Locust Bean (*Parkia fillicoides* Welw) Seeds (*Iru*)

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Authors' contributions

This work was designed and supervised by author BIA. Author OMD did the literature search while the authors developed the manuscript.

Research Article

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ABSTRACT

Aim: *Iru* is a popular West Africa fermented soup condiment which is also consumed without cooking as snack. This product is mainly fermented by *Bacillus* species. The hypolipidemic activities of *Bacillus* spp. isolated from *iru* have not been documented hence the aim of this study.
Place and Duration of Study: *Iru* sample was bought in an open market in Iworoko-Ekiti, Nigeria and transferred to the Laboratory of the Department of Microbiology, Ekiti State University, Nigeria where other studies were carried out. The study was conducted between January and June, 2012.
Methodology: The properties and *in vivo* hypolipidemic potential of *Bacillus* species from *iru* were investigated using standard microbiological and haematological methods.
Results: The cell free extracts of the *Bacillus* spp. did not produce significant inhibition on the selected Gram positive and Gram negative pathogens. Qualitative enzyme screening of the isolates showed all were haemolysin negative. Only *B. subtilis* was positive to gelatinase while all the isolates produced catalase and lipase. The average weight of the animals after inducement of hyper-cholesterolemia ranged between 60.5g - 95.3g. The amount of serum total cholesterol (TC) in the animals ranged between 124.9 mg/dl – 127.4 mg/dl while that of serum triglycerides (TG), high density protein (HDL) and low density protein (LDL) were 122.5 – 155.3 mg/dl, 10.0 – 15.3 mg/dl and 76.6 – 81.0 mg/dl

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respectively. The weights of hyper-cholesterolemia induced rats challenged with different species of *Bacillus* were relatively lower than those in the control group and also differ significantly from the control, at $p < 0.05$. The values of TC, TG, and LDL were highest in the control (saline) group while the values in the treatment group ranged between 121.3 ± 1.5 and 102.3 ± 6.8 mg/dl for TC. The treatment groups recorded lower values of values for TG (104.7 ± 1.6 - 117.4 ± 9.1 mg/dl) and LDL (42.6 ±7.4 - 59.0 ± 10.2 mg/dl) compared to the control. *B. subtilis* had the highest values of TC but least amount of LDL. TG in all the groups was higher than TC, HDL and LDL. The TC/HDL and the LDL/HDL of the animals in the *iru* group was higher than the other treatment groups but lower than the control.
Conclusion: Compared to the control, hypolipidemic activities of *B. lichenliformis* was the best followed by *B. subtilis. Iru* had the least hypo-cholesterolemic effect.

Keywords: Iru; hypolipidemia; Bacillus spp.; probiotics; cholesterolemia; lipoprotein.

1. INTRODUCTION

Iru is a popular soup condiment consumed in Nigeria and other West African countries [1,2]. It is produced from solid state fermentation of African locust bean seeds (*Parkia fillicoides* Welw). Though the product is called different names based on the region of production, the final product is very similar and consumed the same way. It may be eaten immediately after fermentation with the fermenting organisms like yoghurt and other probiotic foods. *Iru* as it is called in the South Western Nigeria is fermented mainly by *Bacillus* species. The species of the genus *Bacillus* isolated from fermented locust bean seeds include: *subtilis, pumillus, lichenliformis, cereus* and [1,3,4,5].

Bacillus species are normal microbiota of the soil [6,7,8]. Their spores and vegetative cells could withstand the harsh condition of the gut of higher animals [7,9] and there is a growing evidence of symbiotic relationship when found in the gut of higher animals [10,11,12,13]. Administration of actively growing bacteria (probiotics) to animals directly or as a feed supplement has been reported to increase the wellbeing of the animals [6,14].

Unlike enterococci, lactobacilli and bifidobacteria, the probiotic properties of genus *Bacillus* has not been extensively reported [13]. In the recent time the probiotic activities of some species of genus *Bacillus* have been documented. *Bacillus clausii, Bacillus subtilis, Bacillus pumillus, Bacillus coagulans* and *Bacillus cereus* have been reported to possess probiotic properties [15,16] and immunostimulatory activity [17,18]. *Bacillus* spp. have recently been evaluated and listed by the European Food Safety Authority as qualified presumption of safety (QPS) and rated as generally regarded as safe (GRAS) [19]. *Bacillus* spp. have been reported to aid the re-establishment of normal microbiota of the gut and prevent its colonization by *Candida* spp. following antibiotics treatment, especially after a shift in the microbial population of the gastrointestinal tract (GIT). Generally, the hypocholesterolemic effect [cholesterol lowering effect] is an attribute of most probiotes.

The objective of the study was to evaluate the hypolipidemic potential of *Bacillus* species isolated from *iru*, a popular soup condiment in Nigeria. The antibacterial spectrum and probiotic properties of the isolates were also described.

2. MATERIALS AND METHODS

2.1 Source of Test Organism

The modified method of Barbosa *et al*. [12] was used to isolate *Bacillus* spp. from freshly fermented locust bean seeds, *iru*. Five gram of ground *iru* sample was suspended in 10 ml distilled water, boiled in sterile bottles with vigorous shaking. The suspension was ten-fold diluted and heated to 65ºC for 45 min. The suspended *iru* sample was again diluted in absolute ethanol (1:1, v/v) and allowed to stand for one hour at room temperature. The sample was serially diluted, plated on Nutrient Agar (Oxoid) and incubated aerobically at 37ºC for 24 h. Representative colonies with varying morphologies were picked and sub-cultured on slants for further characterization and interpreted according to Holt *et al*. [20].

2.2 Animal Experiment

Forty Albino Wistar male rats [5-7 weeks] were obtained from the Pre-clinical Animal House of the University of Ibadan, Nigeria. The animals were acclimatized for five days and fed with grower's mash (TopFeed®) and adequately supplied with distilled water *ad libitum*. The rats were housed in stainless steel cages in a well-ventilated room at about 27° ± 2°C. The lighting regimen was about 13:11 h of light and dark respectively.

All animal management and experimental procedures were performed in strict accordance with the requirements of the National Research Council's Guide for the Care and Use of Laboratory Animals [21].

The method of Bhatia *et al*. [14] was used to induce hyper-cholesterolemia in the rats. After the induction, animals with high serum cholesterol levels were selected for the challenge test. The experimental rats confirmed to be hyperlipidemic were randomly grouped into five treatment groups and administered with the bacteria isolates from *iru* and the *iru* sample as indicated in Table 1. Each of the groups contained five animals. The animals in the control group were given sterile normal saline. All the animals were given the same amount of commercial feed.

The initial body weights of the rats ranged between 21.9 and 25.9 gram. The animals were challenged by gastric intubation with a 0.25 ml solution containing viable *Bacillus* species of known concentration (Table 1) three times daily for two weeks whereas the control group was given the same volume of sterile normal saline. The final body weights of the rats were determined after 2 weeks. The physical character of the animals was centered on the eyes, fur and skin while the behavioral activity such as lethargy, salivation, sleep (sluggishness), stool consistency and tremors was monitored.

Table 1. Concentration of *Bacillus* spp. and amount of *iru* administered on experimental animals

Groups	Probiotics	Concentrations
Group A	*B. subtilis*	2.29 x 10^6 CFU/ml
Group B	*B. pumillus*	2.44 x 10^6 CFU/ml
Group C	*B. lichenliformis*	2.17 x 10^6 CFU/ml
Group D	*Bacillus* sp.	2.53 x 10^6 CFU/ml
Group E	*Iru*	5.0 g of ground *iru* in 20 ml of distilled water

2.3 Preparation and Antibacterial Activities of Cell-Free Culture Supernatant (CFCS)

Bacillus isolates were grown in Nutrient broth at 37ºC in a shaker incubator at 120 rpm for 18-24 hrs. The supernatant was sterilized by membrane filtration. The cells were harvested, washed and standardized according to the method of CLSI [22]. Antibacterial activity of the CFCS was determined by the agar well diffusion method. A 6.0 mm sterile cork borer was used to make a well in the Mueller Hinton agar (Oxoid) seeded with the indicator organism. The well was filled with the cell free supernatants and the zone(s) of inhibition produced was (were) measured with an electronic Vernier caliper after incubation at 37ºC for 24 h.

2.4 Qualitative Enzyme Screening

The detection of some enzymes: catalase, gelatinase, haemolysin and lipase in the test organisms was carried out as described by Olutiola *et al*. [23] and Fawole and Oso [24].

2.5 Simulation of Intestine Condition

The bacteria cells were suspended in Nutrient broth acidified with concentrated HCl to pH 2 and supplemented with 1.0 mg/ml pepsin [to mimic gastric fluid] as described by Barbosa *et al*. [12]. The broth was placed on Mueller Hinton Agar, incubated at 37ºC and observed for growth after 24 h of incubation. The ability of the isolates to grow in the artificial gastric fluid shows that they can survive in the gastrointestinal tract of higher animals.

2.6 Serum Lipid Analyses

The method of Aderiye et al. [25] was used to separate the serum from the whole blood. The serum total cholesterol (STC), high and low density lipoprotein cholesterols (HDL and LDL) and total triglycerides concentrations were analyzed in triplicates by enzymatic kits and standardized reagents using a Reflotron Analyser (Boehringer Mannheim, Germany) The LDL was calculated as described by Johnson et al. [26].

3. RESULTS

The following species of *Bacillus* were isolated and identified as: *B. subtilis, B. pumillus, B. lichenliformis* and *Bacillus* sp. The weight gained by the animals challenged with the different species of *Bacillus* was relatively lower than the control (saline). Animals in *iru* group had the least weight gain followed by those administered with the *Bacillus* spp. Among the treatment groups, animals 'gavaged' with *B. lichenliformis* had the highest weight gain followed by the *B. subtilis* group. The weight gained by the animals in the treatment groups differ significantly from the control (saline water), at $p < 0.05$ (Fig. 1).

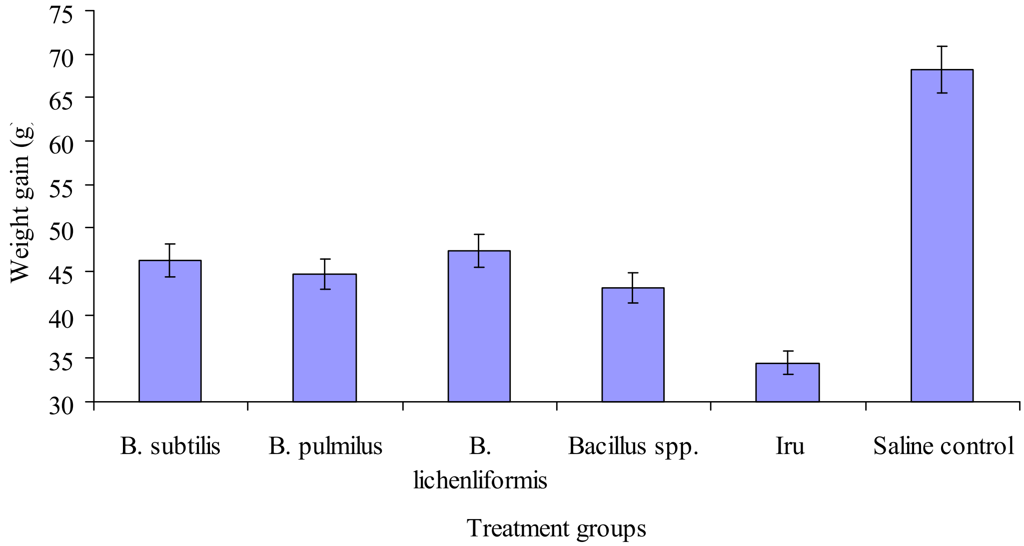

Fig. 1. Effect of administration of probiotes on the weight of the rats challenged by probiotes

The rats in the treatment groups were less active compared to those in the control group immediately after the administration of the 'probiotics'. At the onset of the experiment the furs of the animals in the treatment groups were roughened and languid. None of the animals showed any signs of salivation and the eyes of those in the treatment group were not different from those in the control group. The stool of the animals in all the groups was well formed throughout the experiment. And there was no sign of shiver in any of the animals.

The cell free extracts of the *Bacillus* spp. did not produce significant inhibition on the test organisms. There is clear evidence that these isolates are not bacilliocinogenic. *B. subtilis* and other *Bacillus* spp. did not inhibit any of the test organisms. *B. lichenliformis* had a slight inhibition on the growth of *E. faecalis* and *P. aeruginosa* producing about 2 mm zone of inhibition on each of the isolates [Data not shown]. *B. pumillus* also had a slight inhibitory effect on *E. faecalis.* However, in the other test bacteria antagonistic activity was not noticed when charged against all *Bacillus* isolates (Table 2).

Qualitative enzyme screening of the *Bacillus* species isolated from *iru* sample showed that all the *Bacillus* species are negative to haemolysin while only *B. subtilis* was positive to gelatinase. All the isolates produced catalase and lipase as shown in Table 3.

All the isolates were able to survive the stimulated gastrointestinal tract condition. The weight of the animals after inducement of hyper-cholesterolemia ranged between 60.5g - 95.3g. The amount of serum total cholesterol (TC) in the animals ranged between 124.9 mg/dl – 137.4 mg/dl while that of serum TG, HDL and LDL were 122.5 – 155.3 mg/dl, 10.0 – 15.3 mg/dl and 76.6 – 81.0 mg/dl respectively (Table 4).

Table 2. The antibacterial activities of cell free extracts of *Bacillus* species isolated from *iru*

Isolates	Test organisms	*Antagonistic activity
B. subtilis	*Enterococcus faecalis*	-
	Escherichia coli	-
	Pseudomonas aeruginosa	-
	Salmonella typhi	-
	Staphylococcus aureus	-
B. pumillus	*Enterococcus faecalis*	-
	Escherichia coli	-
	Pseudomonas aeruginosa	-
	Salmonella typhi	-
	Staphylococcus aureus	-
B. lichenliformis	*Enterococcus faecalis*	2
	Escherichia coli	-
	Pseudomonas aeruginosa	2
	Salmonella typhi	-
	Staphylococcus aureus	-
Bacillus spp.	*Enterococcus faecalis*	-
	Escherichia coli	-
	Pseudomonas aeruginosa	-
	Salmonella typhi	-
	Staphylococcus aureus	-

** Determined by zone of inhibition (mm); - : No inhibition zone*

Table 3. Qualitative enzyme screening of the *Bacillus* species isolated from *iru*

Probiotes	Enzymes			
	Catalase	Gelatinase	Haemolysin	Lipase
B. subtilis	+	+	-	+
B. pumillus	+	-	-	+
B. lichenliformis	+	-	-	+
Bacillus spp.	+	-	-	+

+ = Positive; - = Negative

Table 4. Lipid composition of experimental rats after inducement of hyperlipidemia

Serum lipid	Concentration (mg/dl)
Total cholesterol (TC)	124.9 – 127.4
Total glycerides (TG)	122.5 – 155.3
High density lipoprotein (HDL)	10.0 – 15.3
Low density lipoprotein (LDL)	76.6 – 81.0

The values for TC, TG and LDL were highest in the control [saline] group. While the values in the treatment group ranged between 121.3 ± 1.5 and 102.3 ± 6.8 mg/dl for TC. The treatment groups recorded lower values for TG [104.7 ± 1.6 - 117.4 ± 9.1 mg/dl] and LDL (42.6 ±7.4 - 59.0 ± 1.0 mg/dl) compared to the control. Though the group administered with *B. subtilis* had the highest values of TC, it recorded the least amount of LDL. TG values in all the groups were higher than TC, HDL and LDL. The ratio of TC/HDL and the LDL/HDL of the animals in the *iru* group was higher than the other treatment groups but lower in the control.

Compared to the control, the hypolipidemic activities of *B. lichenliformis* was the best followed by *B. subtilis. Iru* exhibited the least effect of all the treatment groups (Table 5).

Table 5. Effect of administration of probiotes on serum lipid of albino rats

Probiotes	TC (mg/dl)	TG (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	TC/HDL	LDL/HDL
B. subtilis	121.3 ± 1.5	113.4 ± 2.2	28.9 ± 3.8	42.6 ± 7.4	4.20	1.47
B. pumillus	110.7 ± 2.9	110.8 ± 1.2	22.1 ± 4.1	59.0 ± 1.0	5.00	2.67
B. lichenliformis	115.4 ± 1.8	112.4 ± 1.5	31.3 ± 1.1	53.4 ± 6.2	3.69	1.71
Bacillus spp.	107.0 ± 2.3	104.7 ± 1.6	19.1 ± 2.6	57.2 ± 5.2	5.60	2.99
Iru	102.3 ± 6.8	117.4 ± 9.1	16.4 ± 2.8	50.6 ± 1.2	6.23	3.09
Control (saline)	127.2 ± 3.3	147.3 ± 3.5	14.3 ± 4.8	79.0 ± 1.2	8.89	5.52

Data are expressed as Mean ± SEM

TC: Total cholesterol, TG: Triglycerides, HDL: High density-lipoprotein, LDL: Low density lipoprotein

4. DISCUSSION

The *Bacillus* isolates used in this study are not bacilliocingenic, which means that they will not disrupt the balance of the gut microbiota. At the same time these organisms are not likely to reduce the load of entero-pathogens. Administration of bacteria belonging to the genus *Bacillus* has beneficial effects in animals under several conditions, like enteritis caused by *Escherichia coli*, *Salmonella enterica*, or *C. perfringens* [12,16]. *Bacillus* spp. may have other subtle means of inhibiting pathogens, producing antibacterial compounds at the stationary phase [27,28].

The weight of the animals after inducement of hyper-cholesterolemia ranged between 60.5g - 95.3g. Several authors reported some beneficial effects of probiotics in the control of both human and animal diseases [12,29]. The weight gained by the animals in the treatment group was lower than the control [saline]. This report contradicts the findings of Cruywagen et al. [30] and Timmerman et al. [31] who recorded an increase in the weight of animals fed with probiotics. All the *Bacillus* spp. were resistant to bile because they grew well under the simulated gastro-acidic conditions. These properties have been reported to be the major determinant of the ability of probiotics to survive in the gut of animals [32,33,34].

Though the group administered *B. subtilis* had the highest values of TC, it recorded the least amount of LDL. TG in all the groups was higher than TC, TG, HDL and LDL. The ratio of TC/HDL and the LDL/HDL of the animals in the *iru* group was higher than the other groups. Probiotic fermented indigenous food has been reported to have positive effects on serum cholesterol levels [35]. The ratio of LDL to HDL in the treatment group ranged between 1.47 and 3.09. The values in the treatment were higher than the control (3.71).

Raised levels of the serum cholesterol are the major cause of cardiovascular diseases, peripheral vascular diseases and atherosclerosis [36]. The mechanism of cholesterol reduction has been proposed to be as a result of bile salt hydrolase enzyme usually secreted by probiotics [32]. This enzyme is used to release some free bile acids by hydrolyzing conjugated bile acids. This activity demands more of cholesterol for further synthesis of the bile salts hence resulting into the reduction of TC and LDL [37].

A significant reduction in the plasma cholesterol and glycerides observed in the treatment group has been reported by Baldwin et al. [38] and Patel *et al.* [34]. Lowering of the serum cholesterol is expected with an increase if the administration of the probiotics takes longer period of time. At varying degrees, different species of *Bacillus* had the ability to reduce serum cholesterol concentrations in the experimental animals and this could lower the risk of disease associated with high cholesterol level and other biochemical parameters [18, 39, 40]. The results of this study show that the species of *Bacillus* isolated from *iru* are capable of reducing the level of cholesterol in the rats. The *in vitro* screening for the presence of the enzyme production in these potential food supplements yields no positive result to virulence. Oral administration of the probiotics to experimental rats showed hypolipidemic effect. It increased the concentration of the HDL and reduced the LDL. The use of these probiotes as feed addictive for both man and animals is recommended. A further study on the mechanism of action(s) of these probiotic organisms from *iru*, their hypolipidemic activities and interactions with the normal microbiota is suggested.

5. CONCLUSION

The hypolipidemic effect of the probiotes varied greatly when administered orally to the experimental rats. The concentration of high density lipoprotein (HDL) increased while the low density lipoprotein (LDL) concentration reduced appreciably. The different species of *Bacillus* had the ability to reduce serum cholesterol concentrations at varying degrees in the experimental animals. Compared to the control (*iru*), hypolipidemic activities of *B. lichenliformis* was the best followed by *B. subtilis*. *Iru* had the least hypo-cholesterolemic effect. It is obvious that the *Bacilli* were more effective in ensuring hypolipidemia. The use of these probiotes as feed addictive for man is very beneficial and therefore recommended.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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