Assessment and characterization of lactic acid bacteria isolated from fermented African oil bean seed (*Pantaclethra macrophylla*) for probiotic application

Abstract

Among the bacteria in fermented foods, lactic acid bacteria play a pivotal role and its main function is to convert carbohydrates and other related raw materials into lactic acid (organic acid). Therefore, the aim of this research was to assess and use lactic acid bacteria isolates obtained from fermented African oil bean seeds as a probiotic. Lactic acid bacteria were isolated using MRS agar and screened for organic acid production and the ability to tolerate some environmental conditions such as temperature, pH, and osmotic pressure. The use of the isolates as probiotic were investigated by screening and determining the antimicrobial inhibitory zone diameter of the isolates obtained against the indicator microorganisms such as *Staphylococcus aureus*, *Bacillus cerus*, *Escherichia coli* and *Listerial monocytogenes*. Result shows that, isolate2, isolate3, isolate5, isolate6, isolate7 and isolate8 were able to produce organic acid. Out of the six bacterial isolates tested for antimicrobial activities against the indicator microorganisms, isolate2, isolate3, isolate6 and isolate8 were able to show antimicrobial activities against *Staphylococcus aureus*, *Bacillus cerus, Escherichia coli* and *Listerial monocytogenes* using different dilutions (10^{-1} to 10^{-3}) of the isolates. The isolates were able to grow at temperature of 37 and 45, pH of 2.5, 3.0, 3.5 and 4.0 and finally the osmotic pressure of 1.0, 1.5, 2.5 and 5.0 % w/v NaCl. The isolates were identified as *Lactobacillus* species and can be used as probiotic.

Introduction

Lactic acid bacteria plays an important role in the acidification of raw material through the production of organic acids **(Leroy and Devuyst, 2004; Shivran and Vishwanath, 2012; Ezea *et al.*, 2014)**. Isolation and assessment of microorganisms from natural environment such as fermented foods have always been the most powerful means for obtaining useful starter cultures for Industrial fermentation (Ezea *et al*., 2014).

Probiotic bacteria sold mainly in fermented foods and dairy products play a predominant role as a carrier of probiotics **(Heller, 2001; Subitsha and Sabu, 2021)**. Probiotic strains found in fermented foods and dairy products are compatible to promote the positive health impact in human and animal in lactose intolerance, urinary tract infection in woman and traveler's diarrhea **(Subitsha and Sabu, 2021; Jomehzadeh *et al*., 2020)**.

Aside treatment of infection, research on lactic acid bacteria has confirmed how specific strains possess probiotic properties and impart unique sensory characteristics to food products **(Raphael *et al.*, 2020)**. Lactic acid bacteria is employed in many food fermentation and have been recognize for it's biopreservative attributes **(Ezea *et al*., 2014; Raphael *et al.*, 2020; Obadina *et al.*, 2006; Ngene *et al*., 2019).**

During fermentation, lactic acid bacteria produce organic acids and other metabolites that enhance flavor development in food, prevent spoilage, and are thus very useful in many

applications especially in food and dairy industry **(Hati *et al.*, 2013).** Distinct nutritional properties of lactic acid bacteria couple with enhanced adhensional adaptative features enable the bacteria to easily thrive in different environment such as in dairy- based food, fermented food, vegetables and other foods that contain salt **(Bintsis, 2018; Raphael *et al.*, 2020)**.

In the recent time, consumers are concerned about the synthetic chemical used as preservatives in food **(Soomro *et al.*, 2002).** A solution to this is the use of antimicrobial metabolites of fermentative microorganisms or probiotics. Many antimicrobial chemical have been used for a long time without any known adverse effects. Many of the organic compounds which have stirred interest are antimicrobial metabolites of bacteria associated with fermented foods. Therefore, the aim of this research was to assess and use lactic acid bacteria isolates isolated from African oil bean as probiotics.

Keywords: Lactic acid bacteria, fermentation, African oil bean, organic acid, environmental tolerance.

Materials and Methods

Collection and preparation samples

Samples of fermented African oil bean seeds (*Pantaclethra macrophylla*) were sourced randomly from two local markets at Nsukka, Enugu State, Nigeria from five local dealers. A 1 g of the fermented African oil bean seeds was weighed, suspended in 9 ml aliquot of distilled water, mashed and mixed properly. Thereafter, a ten-fold serial dilution was made.

Media preparation

A 67.1 g of De Man Rogosa Sharpe (MRS) agar was dissolved in 1000 ml of distilled water according to manufacturer's instruction. The medium was gently heated to homogenize and was sterilized by autoclaving at 15 Psi (121°C) for 15 minutes. The medium was cooled and dispensed into sterile petridish plates. MRS broth was prepared by dissolving 67.1 g of the medium in 1000 ml distilled water and filter to remove the agar prior to sterilization.

Isolation of lactic acid bacteria

Ten-fold serial dilutions of each of the prepared samples were made. A 0.1 ml of each dilution was inoculated into MRS agar by spread plate method and incubated at 37°C for 48 hours. After incubation, colonies were purified by successive streaking on MRS agar plates and maintained on MRS agar slants, stored at 4 ° C and subculture at intervals.

Screening for Organic acid producing isolates

Organic acid producing isolates were screened using 0.6 % $CaCO_3$ (w/v) incorporated in ~~de Man Rogosa Sharpe~~ (MRS). MRS agar containing 0.6 % $CaCO_3$ was prepared and the medium was sterilized in autoclave and allowed to cool before dispensed into a sterile Petri dish plates. A spot of the isolates were made on the surface of 0.6 % $CaCO_3$ agar and incubated at 37°C for 24 hours. Acid producing bacteria were identified by the clear zone around the isolates.

Screening for antimicrobial activities

Antimicrobial activities to all the indicator organisms (*Staphylococcus aureus, Bacillus cerus, Escherichia coli* and *Listeria monocytogenes*) were conducted using agar spot test method describe by **Schillinger and Lucke (1989).** Overnight cultures of the isolates in MRS broth were spotted (2 ul) onto MRS agar containing 0.2 % (w/v) glucose and seeded with 100 ul of the indicator microorganisms. The plates were incubated for 24 hours at 37 ° C. Thereafter, the plates were examined for the antimicrobial activities by the formation of zone of clearance around the spotted isolates.

Determination of inhibitory activities by the isolates

The inhibitory activities of the isolates obtained from fermented African oil bean seeds were determined using agar well diffusion method. MRS agar was prepared and seeded with 100 ul of the indicator strains and allowed to solidify. Five Wells were bored on each MRS agar plates using a sterile 6 mm diameter cork borer to accommodate the isolates. A 200 ul of the MRS broth containing overnight culture of different dilutions (10^{-1} to 10^{-3}) of the isolates were introduced into each well. The plates were kept in the refrigerator for 30 minutes and then incubated at 37 ° C for 24 hours. Thereafter, the diameter of the clear zones was measured.

Determination of the environmental tolerant on the isolates

Environmental tolerant on the isolates were determined according to the method of **De Man *et al.* (1960)**. MRS agar was incorporated with 0.17 g/l bromocresol purple as pH indicator. A lowering of pH would change the medium from purple to yellow and was used to indicate cell growth because of lactic acid production. The isolates were subjected to various temperature ranges of 20, 30, 37, 45, 50 and 55 ° C. For acid tolerance the isolates were subjected to pH values of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0. The isolates were also subjected to osmotic tolerance by varying the percentage of NaCl; 1.0, 1.5, 2.5, 5.0, 7.0, 10, 12, and 14 (% w/v). After subjecting to the above environmental parameters, the plates incubated at 37 ° C for 48 hours. The color change and turbidity of each isolate was noted as a simple indicator of growth and was reported as positive or negative.

Identification of the isolates

The isolates obtained from fermented African oil bean seeds were identified using microscopy, biochemical test and fermentation profile method of identification according to Bergey's manual of determinative bacteriology **(Holt *et al*., 1994).**

Results

Screening for organic acid production by the bacterial isolates

Table 1 shows the ten bacterial isolates from fermented oil bean seeds. Out of the ten isolates; isolate2, isolate3, isolate5, isolate6, isolate7 and isolate8 were able to produce clear zone on $CaCO_3$ which indicates production of organic acids. Isolate1, isolate4, isolate9 and isolate10 had no clear zone with the $CaCO_3$, which indicates no organic acid production.

Table 1: Screening for organic acid production by the bacterial isolates

S/no	Bacterial Isolates	Clear Zones on 0.6% $CaCO_3$
1	Isolate1	-
2	Isolate2	++
3	Isolate3	++
4	Isolate4	-
5	Isolate5	++
6	Isolate6	++
7	Isolate7	++
8	Isolate8	++
9	Isolate9	-
10	Isolate10	-

Keys: ++ = Clear zone on $CaCO_3$,
- = No clear on $CaCO_3$.

Screening for antimicrobial activity by the bacterial isolates

Out of the six bacterial isolates tested for antimicrobial activities, isolate2, isolate3, isolate6 and isolate8 were able to show antimicrobial activities against the indicator organisms *(Staphylococcus aureus, Escherichia coli, Listeria monocytogenes* and *Bacillus cerus*) whereas, the isolate5 and isolate7 showed no antimicrobial activities against the indicator strains (table 2). Clear zones around the isolates indicated antimicrobial activities against the indicator strains whereas, no clear zones around the isolates indicated no antimicrobial activities against the indicator strains.

Table 2: Screening for antimicrobial activity by the bacterial isolates

Indicator strains	Isolate2	Isolate 3	Isolate5	Isolate6	Isolate7	Isolate8
Staphylococcus aureus	++	++	-	++	-	++
Escherichia coli	++	++	-	++	-	++
Listerial monocytogens	++	++	-	++	-	++
Bacillus cerus	++	++	-	++	-	++

Keys: ++ = Clear Zone by the isolates on the indicator strains
- = no clear zone on the indicator strains

The inhibitory zone diameter of different dilutions of the bacterial isolates

The inhibitory zone diameter of 10^{-1} dilutions of the bacterial isolates against Staphylococcus *aureus, Escherichia coli, Listeria monocytogenes* and *Bacillus cerus* as the indicator strains shows that isolate2, isolate3, isolate6 and isolate8 were able to produce a significant clear zone diameters with10^{-1} dilutions against the indicator strains (table 3). Table 4 shows the inhibitory zone diameter on 10^{-2} dilutions of the bacterial isolates from fermented oil bean seeds. Isolate2, isolate3, isolate6 and isolate8 showed a significant inhibitory zone diameter against the indicator strains; Staphylococcus *aureus, Escherichia coli, Listeria monocytogenes* and *Bacillus cerus.* Determination of inhibitory zone diameter on 10^{-3} dilutions of the bacterial isolates from

fermented oil bean seeds shows that isolate2 had inhibitory zone only on *Staphylococcus aureus* and *Escherichia coli* whereas, *Listeria monocytogenes* and *Bacillus cerus* had no inhibitory zone diameter with the isolate2 on 10^{-3} dilutions. Isolate3 had inhibitory zone only on *Escherichia coli* while isolate6 and isolate8 had inhibitory zone diameter against *Staphylococcus aureus* and *Escherichia coli* . *Listeria monocytogenes* and *Bacillus cerus* showed no inhibitory zone diameter with the isolates at 10^{-3} (table 5).

Table 3: Determination of the inhibitory zone diameters (Mm) on 10^{-1} dilutions of the bacterial isolates

Indicator strains	Isolate2 (Mm)	Isolate3 (Mm)	Isolate6 (Mm)	Isolate8 (Mm)
Staphylococcus aureus	7.5 ± 0.3	5.7 ± 0.6	10.1 ± 0.8	10.8 ± 1.0
Escherichia coli	9.2 ± 0.5	6.1± 0.4	11.0 ± 0.9	10. 4 ± 0.8
Listerial monocytogens	5.6 ± 0.2	3.5± 0.4	8.5 ± 0.5	5.9 ± 0.7
Bacillus cerus	4.5 ± 0.2	3.6 ± 0.1	5.2 ± 0.6	3.2 ± 0.1

Key: Mm (inhibitory zone diameters)

Table 4: Determination of the inhibitory zone diameters (Mm) on 10^{-2} dilutions of the bacterial isolates

Indicator strains	Isolate2 (Mm)	Isolate3 (Mm)	Isolate6 (Mm)	Isolate8 (Mm)
Staphylococcus aureus	5.0 ± 0.2	4.0 ± 0.3	8.5 ± 0.9	9.5 ± 0.2
Escherichia coli	6.5 ± 0.3	5.1 ± 0.4	9.8 ± 0.7	8.6 ± 0.7
Listerial monocytogens	3.6 ± 0.4	3.0 ± 0.1	5.5 ± 0.6	5.0 ± 0.5
Bacillus cerus	3.1 ± 0.5	3.0 ± 0.3	4.5 ± 0.3	3.2 ± 0.6

Key: Mm (inhibitory zone diameters)

Table 5: Determination of the inhibitory zone diameters (Mm) on 10^{-3} dilutions of the bacterial isolates

Indicator strains	Isolate2 (Mm)	Isolate3 (Mm)	Isolate6 (Mm)	Isolate8 (Mm)
Staphylococcus aureus	3.2 ± 0.2	-	5.5 ± 0.4	4.3 ± 0.6
Escherichia coli	4. 5 ± 0.5	3.2 ± 0.3	4.0 ± 0.5	3.5 ± 0.5
Listerial monocytogens	-	-	-	-
Bacillus cerus	-	-	-	-

Keys: Mm (inhibitory zone diameters)

- = No inhibitory zone diameters

The effect of different environmental tolerant by the bacterial isolates

Table 6 shows the temperature tolerant on the growth of the bacterial isolates from fermented oil bean seeds. Isolates2, isolate3, isolate6 and isolate8 had growth at the temperature of 37°C and 45°C. All the bacterial isolates had no growth at the temperature of 20°C, 25°C, 50°C and 55°C except isolate6 and isolate8 that had growth at 30°C. Determination of acid tolerant on the bacterial isolates shows that isolate2, isolate3, isolate6 and isolate8 had growth on pH 2.5, 3.0, 3.5 and 4.0 whereas, pH 1.0, 1.5 and 2.0 had no growth in all the isolates except isolate2 and isolate8 that had growth on pH 2.0 (table 7). Determination of salt tolerant on the bacterial isolates shows that isolate2, isolate3, isolate6 and isolate8 had growth on 1.0, 1.5, 2.5 and 5.0 % (w/v) NaCl whereas, all the isolates had no growth 6.5, 10.0, 12.0 and 14.0 % w/v NaCl (table 8).

Table 6: Effect of temperature on the growth of the bacterial isolates

Temperature (^{0} C)	Isolate2	Isolate3	Isolate6	Isolate8
20	-	-	-	-
25	-	-	-	-
30	-	-	++	++
37	++	++	++	++
45	++	++	++	++
50	-	-	-	-
55	-	-	-	-

Keys: ++ = Growth
- = No growth

Table 7: Acid tolerant on the growth of the bacterial isolates

pH	Isolate2	Isolate3	Isolate6	Isolate8
1.0	-	-	-	-
1.5	-	-	-	-
2.0	++	-	-	++
2.5	++	++	++	++
3.0	++	++	++	++
3.5	++	++	++	++
4.0	++	++	++	++

Keys: ++ = Growth
- = No growth

Table 8: Salt tolerant on the growth of the bacterial isolates

NaCl (% w/v)	Isolate2	Isolate3	Isolate6	Isolate8
1.0	++	++	++	++
1.5	++	++	++	++
2.5	++	++	++	++
5.0	++	++	++	++
6.5	-	-	-	-
10.0	-	-	-	-
12.0	-	-	-	-
14.0	-	-	-	-

Keys: ++ = Growth
- = No growth

Table 9 shows microscopy, biochemical characteristics and the sugar fermentation profile of the four selected isolates from the fermented oil bean seeds. The four isolates were Gram-positive rod, catalase, indole and oxidase negative. The four isolates were able to ferment Arabinose, galactose, lactose, sucrose, salcin and trehalose with gas production which tentatively confirmed the isolates as *Lactobacillus* strains.

Table 9; microscopic, biochemical characteristics and fermentation profile of the four selected isolates of *Lactobacillus* spp.

Isolates	Gra	Cat	Indo	Oxi	Arabi	Galac	Lact	Man	Suc	Sal	Treha
Isolate2	+ve rod	-ve	-ve	-ve	+G	+G	+G	No	+G	+G	+G
Isolate3	+ve rod	-ve	-ve	-ve	+G	+G	+G	No	+G	+G	+G
Isolate6	+ve rod	-ve	-ve	-ve	+G	+G	+G	No	+G	+G	+G
Isolate8	+ve rod	-ve	-ve	-ve	+G	+G	+G	No	+G	+G	+G

Keys: +ve rod = gram positive rod, -ve = negative, +G = fermentation with gas production, No = no fermentation, Gra = gram staining reaction, Cat = catalase. Indo = indole, Oxi = oxidase, Arabi = arabionose, Galac = galactose, Lact = lactose, Man = mannose, Suc = sucrose, Sal = salcin, Trehal = trehalose

Discussion

The isolates; isolate2, isolate3, isolate6 and isolate8 were Gram- positive rod and catalase negative and were able to grow in the presence of 5.0 % (w/v) NaCl, tolerated pH of 2.5 and

grown at high temperature of 45°C. Sugar fermentation profile confirmed that all the four isolates were likely to be *Lactobacillus* strains. This is in agreement with **Bazireh *et al*. (2020)** who isolated noval probiotic *Lactobacillus* and *Enterococcus* strains from human salivary and fecal sources. This is also in agreement with **Negene *et al*. (2012)** who reported similar results during screening of some Lactic acid bacteria isolates isolated from selected Nigeria fermented foods for vitamin production. The identification is also in line with *Ezea et al. (2014)* who studied and used Lactobacillus isolates obtained from fermented soybean milk as probiotics.

This study revealed the selection of Lactic acid bacteria from traditional fermented food. Lactic acid bacteria were isolated from fermented African oil bean seeds. The isolates tolerated high temperature, osmotic stress from NaCl, low pH and produced organic acid. The isolates exhibited antimicrobial activities against the indicator organisms such as Staphylococcus *aureus, Escherichia coli, Listeria monocytogenes* and *Bacillus cerus.* This is agreement with **Somashekaraiah *et al*. (2019)** who reported and proved that Lactic acid bacteria isolates isolated from fermenting coconut palm nectar (Neera) exhibited promising probiotic properties and seen favorable for use in functional fermented food as preservatives. **Somashekaraiah *et al*. (2019)** reported that Lactic acid bacteria isolated from the fermenting coconut palm nectar showed optimum growth and also substained osmotic stress at different NaCl concentrations.

The isolates were further tested for antimicrobial activities. The isolate2, isolate3, isolate6 and isolate8 showed antimicrobial activities against the indicator organisms. The results obtained showed the role of organic acid for the antagonistic activity of the isolates from fermented African oil bean seeds. The production of organic acid reduces the pH of the media, which lead to inhibition of the indicator organisms by destruction of the vital cell function **(Kivanc and Yilmaz, 2011)**. Organic acid is one of the antimicrobial metabolites associated with probiotics when used as starter culture **(Henning *et al.*, 2015).**

The inhibitory effect of Lactic acid bacteria isolates were found against the indicator strains (*Staphylococcus aureus, Escherichia coli, Listeria monocytogenes* and *Bacillus cerus*) using agar well diffusion. This result is in agreement with **Zommiti *et al*. (2018)** who reported broad-spectrum antimicrobial activities against *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes* and *Bacillus cerus, Enterococcus faecalis* and *Candida albicans* during evaluation of probiotic properties and safety of *Enterococcus faecium* isolated from artisanal Tunisia meat. **Bazireh *et al*. (2020)** reported similar zone of inhibition showing antimicrobial activity using agar well diffusion during isolation of novel probiotic *Lactobacillus* and *Enterococcus* strains from human salivary and fecal sources. This present research is in agreement with **Karami *et al*. (2017)** who reported similar zone of inhibition diameter during isolation of probiotic Lactobacillus from local dairy and evaluating their antagonistic effect on pathogens. The work of **Ezea *et al*. (2014)** is also in agreement with the present research. They reported similar zone of inhibition diameter during studies and use of lactobacillus isolates obtained from fermented soybean milk as probiotic. **Reuben *et al.* (2019)** reported in accordance with our research work with similar zone of inhibition activity of potential Lactic acid bacteria strain from broiler gastrointestinal tract against pathogenic bacteria by agar well diffusion technique during isolation, characterization and assessment of Lactic acid bacteria toward the selection as poultry probiotics. These reports with our findings suggested that the broad antimicrobial effect of Lactic acid bacteria are most often as the result of organic acid production.

Conclusions

Fermented African oil bean seeds were good source for the isolation of Lactic acid bacteria. The *Lactobacillus* Spp isolated from the fermented oil bean were able to produce organic with an adverse environmental tolerance and had good antimicrobial activities against the indicator microorganisms. Therefore the lactic acid bacteria isolates obtained from the fermented oil bean seeds can be used as a probiotic for industrial application

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