

Assessment and characterization of lactic acid bacteria isolated from fermented African oil bean seed (*Pentaclethra 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 probiotics. 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 probiotics was investigated by screening and determining the antimicrobial inhibitory zone diameter of the isolates obtained against the indicator microorganisms such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Listeria monocytogenes*. Results show 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 cereus*, *Escherichia coli* and *Listeria monocytogenes* using different dilutions (10^{-1} to 10^{-3}) of the isolates. The isolates were able to grow at temperatures of 37°C and 45°C; pH of 2.5, 3.0, 3.5 and 4.0; 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 probiotics.

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

Introduction

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

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

Aside from the 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 recognised for their 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 the food and dairy industry (**Hati *et al.*, 2013**). Distinct nutritional properties of lactic acid bacteria couple with enhanced adhesional 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 recent times, consumers have been concerned about the synthetic chemicals 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 chemicals 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.

Materials and Methods

Collection and preparation of samples

Samples of fermented African oil bean seeds (*Pentaclethra macrophylla*) were sourced randomly from two local markets at Nsukka, Enugu State, Nigeria from five local dealers. One gram of the fermented African oil bean seeds was weighed, suspended in a 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 subcultured at intervals.

Screening for Organic acid producing isolates

Organic acid producing isolates were screened using 0.6 % CaCO₃ (w/v) incorporated in (MRS). MRS agar containing 0.6 % CaCO₃ was prepared and the medium was sterilized in an autoclave and allowed to cool before dispensed into sterile Petri dish plates. A spot of the isolates were made on the surface of 0.6 % CaCO₃ 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 cereus*, *Escherichia coli* and *Listeria monocytogenes*) were conducted using agar spot test method described 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 a 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 a clear zone on CaCO_3 which indicates the 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 bacteria 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 CaCO₃,
 - = No clear on CaCO₃.

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 cereus*) 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 bacteria isolates

Indicator strains	Isolate2	Isolate3	Isolate5	Isolate6	Isolate7	Isolate8
<i>Staphylococcus aureus</i>	++	++	-	++	-	++
<i>Escherichia coli</i>	++	++	-	++	-	++
<i>Listeria monocytogenes</i>	++	++	-	++	-	++
<i>Bacillus cereus</i>	++	++	-	++	-	++

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 bacteria isolates

The inhibitory zone diameter of 10⁻¹ dilutions of the bacterial isolates against *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Bacillus cereus* as the indicator strains shows that isolate2, isolate3, isolate6 and isolate8 were able to produce a significant clear zone diameters with 10⁻¹ 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 cereus*. 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 cereus* 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 cereus* 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 bacteria isolates

Indicator strains	Isolate2 (Mm)	Isolate3 (Mm)	Isolate6 (Mm)	Isolate8 (Mm)
<i>Staphylococcus aureus</i>	7.5 ± 0.3	5.7 ± 0.6	10.1 ± 0.8	10.8 ± 1.0
<i>Escherichia coli</i>	9.2 ± 0.5	6.1 ± 0.4	11.0 ± 0.9	10.4 ± 0.8
<i>Listeria monocytogenes</i>	5.6 ± 0.2	3.5 ± 0.4	8.5 ± 0.5	5.9 ± 0.7
<i>Bacillus cereus</i>	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 bacteria isolates

Indicator strains	Isolate2 (Mm)	Isolate3 (Mm)	Isolate6 (Mm)	Isolate8 (Mm)
<i>Staphylococcus aureus</i>	5.0 ± 0.2	4.0 ± 0.3	8.5 ± 0.9	9.5 ± 0.2
<i>Escherichia coli</i>	6.5 ± 0.3	5.1 ± 0.4	9.8 ± 0.7	8.6 ± 0.7
<i>Listeria monocytogenes</i>	3.6 ± 0.4	3.0 ± 0.1	5.5 ± 0.6	5.0 ± 0.5
<i>Bacillus cereus</i>	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)
<i>Staphylococcus aureus</i>	3.2 ± 0.2	-	5.5 ± 0.4	4.3 ± 0.6
<i>Escherichia coli</i>	4.5 ± 0.5	3.2 ± 0.3	4.0 ± 0.5	3.5 ± 0.5
<i>Listeria monocytogenes</i>	-	-	-	-
<i>Bacillus cereus</i>	-	-	-	-

Keys: Mm (inhibitory zone diameters)

- = No inhibitory zone diameters

The effect of different environmental tolerant by the bacteria isolates

Table 6 shows the temperature tolerance 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 bacteria 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 bacteria 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 bacteria 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 at 6.5, 10.0, 12.0 and 14.0 % w/v NaCl (table 8).

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

Temperature (°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 bacteria 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 bacteria 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 rod	+ve	-ve	-ve	-ve	+G	+G	+G	No	+G	+G	+G
Isolate3 rod	+ve	-ve	-ve	-ve	+G	+G	+G	No	+G	+G	+G
Isolate6 rod	+ve	-ve	-ve	-ve	+G	+G	+G	No	+G	+G	+G
Isolate8 rod	+ve	-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 = arabinose, 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 a high temperature of 45°C. The sugar fermentation profile confirmed that all four isolates were likely to be *Lactobacillus* strains. This is in agreement with **Bazireh et al. (2020)** who isolated novel 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 cereus*. This is in 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 were 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 sustained 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 in the antagonistic activity of the isolates from fermented African oil bean seeds. The production of organic acid reduces the pH of the media, which leads 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 a 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 cereus*) 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 cereus*, *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 a 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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