



Case Study

Isolation of *Lactobacillus* species from fermented *Parkia biglobosa* seed and screening for their probiotic activity

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(Manuscript received 24 December 2020; accepted for publication 10 May 2021)

Abstract. Food fermentation has been a tradition for decades due to its immense benefits. Lactic acid bacteria are known to possess probiotic potential due to various chemical antimicrobial substances they produce. This research was aimed at isolating *Lactobacillus* spp. from locally fermented locust beans and screening the isolates for their probiotic potential. A total of 20 samples of fermented locust beans (*P. biglobosa*) were collected and processed. The samples were inoculated onto de-Mann Rogosa Sharpe (MRS) media and incubated anaerobically. The isolates with characteristic colonial morphology of *Lactobacillus* sp. were characterized using Gram staining and biochemical tests. *Lactobacillus* spp. isolates were screened in-vitro for probiotic potential using the following parameters, tolerance of low pH, bile tolerance and antimicrobial activity. Four isolates of *Lactobacillus* species consisting of *Lactobacillus fermentum* and *Lactobacillus plantarum* were isolated from different fermented locust beans. All isolates showed tolerance to low pH and bile concentration of 3.04 and 0.3%, respectively. The *Lactobacillus* species isolates showed varying antibacterial activity against all test bacteria with isolate S06 (*L. plantarum*) showing the highest degree of antibacterial activity. In conclusion, the *Lactobacillus* species isolated from fermented locust beans can serve as probiotic candidate.

Keywords: antimicrobial activity, fermentation, local locust beans, potential, probiotic

Introduction

Parkia biglobosa is a tropical plant known to be indigenous to Africa and was named after its discoverer Mungo Park (a Scottish physician) by Robert Brown (Ajayi et al., 2015). It is well-known and used in Nigeria and other African countries including Senegal, Mali, Sudan, Ghana, Guinea Bisau and Cameroon amongst others (Olujobi, 2012). Fermented *Parkia biglobosa* seed is known by different names in various Nigerian dialects: iru in Yoruba, dawa dawa in Hausa and daddawa in Igbo (Ojewumi et al., 2016). It is known to be high in nutritional value, hence a reason for its use as a food source especially in developing or underdeveloped countries as it serves as a cheap source for protein. It is known for a considerable amount of proximate constituent in its different parts (i.e. crude fibre, crude protein, crude fat and moisture content) vitamins and minerals (Olujobi, 2012; Ojewumi et al., 2016).

Parkia biglobosa seed is traditionally fermented to produce a local condiment (iru dadawa or ogiri) by moist solid state fermentation known for its overtone production of ammonia due to hydrolysis of amino acids during fermentation. A process known to be a classical alkaline fermentation with pH ranging from 6.0-8.39. This is responsible for the fermented locust bean organoleptic property resulting in characteristic taste and

sweet aroma when used to cook local dishes (Adelekan and Nwadiuto, 2012).

Probiotics are defined by WHO as live microbial food supplements which, when administered in adequate amounts, confer a health benefit on the host (Shehata et al., 2016). Among the usually used microorganisms, lactic acid bacteria (LAB) are regarded as a major group of probiotic bacteria. Probiotics are non-pathogenic, technologically suitable for industrial processes, acid tolerant, bile tolerant and produce antimicrobial substances (Mojgani et al., 2015). They are classified as generally recognized as safe (GRAS) microorganisms because of their long and safe use as starter cultures in fermented products (Shehata et al., 2016).

Probiotics are a promising natural source of conferring health benefits. They can be sourced from human, animal and some fermented food products. It is important to note that although there are different genera of microorganisms with probiotic potential, they are strictly strain specific and a consideration in their biosafety. The following are genera of bacteria with recognized probiotic potential *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Streptococcus*, all of which belong to a general group, lactic acid bacteria (LAB). Other genera are *Bifidobacterium*, *Saccharomyces*, *Escherichia* and *Bacillus* (Sabina, 2014).

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Lactobacillus species and other LAB isolated from fermented seeds and foods have been screened for their probiotic potential by different researchers. Pundir et al. (2013) reported that LAB isolated from different food samples exhibited excellent probiotic potentials. Species of *Lactobacillus* isolated from fermented curly kale juice (Szutowaska and Gwiazdowska, 2021), Brazilian regional ovine cheese (Meira et al., 2012) and fermented African locust bean seeds (Nwagu et al., 2020) all had promising results as probiotic candidates.

LAB have become the most widely used probiotics for medicine and food production. As probiotics, firstly, the microorganisms must be non-pathogenic and non-toxigenic; secondly, they should withstand external and internal environments, including gastric acid and bile salts. In addition, the adhesive ability of LAB is a primary consideration in the selection of probiotics (Saad et al., 2013).

The sources of LAB are diverse. The natural environment, intestinal tracts of mammals, and fermented foods are all sources of LAB. Fermented dairy products, especially fermented milk and cheese, are the main sources of LAB (Ji et al., 2015; Stojanovski et al., 2021). Fermented meat products, especially fermented sausages, are also an excellent source. Some studies have isolated and identified LAB from fermented sausages (Han et al., 2017; Stojanovski et al., 2020).

This research was carried out to determine the probiotic potential of *Lactobacillus* species isolated from fermented *Parkia biglobosa* seeds.

Material and methods

Sample collection

A total of 20 samples of locally fermented *Parkia biglobosa* beans were collected from within and outside Zaria, Kaduna central market and stored in the refrigerator prior to use.

Isolation of *Lactobacillus* species

For each of the 20 samples, 10 g was appropriately weighed and aseptically transferred into labelled conical flask containing 90 mL of sterile peptone water and homogenized. A sterile syringe was used to transfer 1 mL into 9 mL sterile peptone water to obtain 10^{-1} dilution. Further dilutions were carried out serially from 10^{-1} to 10^{-4} , then 0.1 mL aliquot of dilution 10^{-4} were spread onto de-Mann Rogosa Sharpe (MRS) media (Himedia, USA) and incubated at 37°C or 48 hrs (Shehata et al., 2016).

Characterization of *Lactobacillus* species

The isolates were characterized based on their colonial morphology on MRS medium, Gram reaction and biochemical reactions (Catalase, Methyl-Red, Voges-Proskauer, Citrate Utilization and Sugar Fermentation Test) (Cowan and Steel, 2003).

Evaluation of the isolates probiotic ability

Preparation of phosphate buffer saline solution: Phosphate saline solution was prepared by dissolving 2 phosphate buffer

saline tablets into 200 mL distilled water and dispensed into 3 separate conical flasks. Dilute 0.1M of NaOH and 0.1M HCl was used to adjust the pH of the phosphate buffer contained in a separate conical flask to pH 7.22 and 3.04, respectively intended for use for the physiological test. The third was adjusted to pH 6.5. All were autoclaved at 121°C at 15 psi for 15 min.

Acid tolerance test: The acid tolerance test was performed by the viable plate count method as described by Maragkoudakis et al. (2006) with modification. Overnight bacterial cell culture grown in MRS broth at 37°C were harvested by centrifuging at 10,000 rpm at 4°C for 10 min. Supernatant was discarded and cell suspension was washed twice with phosphate buffer saline (PBS) (Sigma-Aldrich, USA) of pH 7.22 and re-suspended in PBS of pH 3.04 for 3 hrs. Aliquot of the cell suspension was plated on sterile MRS agar plates both at 0 hr and 3 hrs. Plates were incubated anaerobically at 37°C for 24 hrs.

Bile tolerance test: Ability of the isolates to tolerate bile was also determined by the viable plate count method. Overnight MRS broth culture of the bacterial cells were harvested at 10,000 rpm at 4°C for 10 min washed twice with PBS of pH 7.22 before being resuspended in PBS solution containing 0.3% Oxgall bile (Sigma-Aldrich, USA). Cell aliquot was plated at 0 hr and 4 hrs on sterile MRS agar plate and incubated at 37°C for 24 hrs (Maragkoudakis et al., 2006).

Antimicrobial activity of the *Lactobacillus* isolates

The Oxford cup assay was used as reported by Wang et al. (2009). Test bacteria that were used for the antibacterial activity were *Pseudomonas aeruginosa* and *Salmonella* spp. *Lactobacillus* species isolates were subcultured prior to use in the experiments. Isolates were incubated on MRS medium and incubated anaerobically. The bacterial cultures were centrifuged at 10,000 rpm for 10 min. Supernatants were filtered through a 0.45-µm filter paper. The inoculum of indicator (pathogen) were each seeded on the surface of sterile Muller-Hinton plates. Cork borer was heat sterilized and used to bore well on the agar plate. About 10 drops of cell free culture suspension (CFCs) were transferred into well using sterile syringe. Chloramphenicol disc (Oxoid, UK) was aseptically placed at the center of each plate to serve as a positive control while the plate for the negative control was done using sterilized normal saline. The plates were incubated for 18-24 hrs at 37°C. Antibacterial activity was indicated by the observation of zones of inhibition. The diameter of inhibition zones were measured in millimeter (mm). Zone diameters ≥ 5 mm were considered positive for inhibitory activity while zone diameters ≤ 4 mm were considered negative for inhibitory activity.

Results

Isolation and identification *Lactobacillus* species

The cultural, microscopic and the biochemical characteristics of the different isolates of *Lactobacillus* species from different samples of locally fermented locust beans

are shown in Table 1. Four isolates from different samples had the characteristic colonial appearance of *Lactobacillus* species on MRS Agar. Further characterization of these

isolates microscopically and biochemically revealed that two of the isolates were *L. fermentum* while the remaining two isolates were *L. plantarum*.

Table 1. Cultural, microscopic and biochemical characteristics of bacterial isolates from fermented locust beans

Isolates code	Cultural characteristic	Gram reaction	Cat	MR	VP	Cit	Suc	Ara	Gal	Glu	Lac	Dex	Inference
S01	Yellow, irregular moist	G+ rods	-	+	-	-	+	+	+	+(AG)	+	+	<i>L. fermentum</i>
S02	Milky white, raised, moist	G+ rods	-	+	-	-	+	+	+	+	+	+	<i>L. plantarum</i>
S06	Milky white, smooth, moist	G+ rods	-	+	-	-	+	+	+	+	+	+	<i>L. plantarum</i>
S08	Whitish, flat and moist	G+ rods	-	+	-	-	+	+	+	+(AG)	+	+	<i>L. fermentum</i>

Key: G+= Gram Positive, Cat= Catalase, MR= Methyl Red, VP= Voges-Proskauer, Cit= Citrate utilization, Suc= Sucrose fermentation, Ara= Arabinose fermentation, Gal= Galactose fermentation, Glu= Glucose fermentation, Lac= Lactose fermentation, Dex= Dextrose fermentation, AG= Acid and Gas production.

Physiological characterization of the *Lactobacillus* species isolated

The *Lactobacillus* species isolated were screened for their ability to survive the simulated condition of the gastrointestinal tract, which included ability to tolerate low pH and bile which are characteristics of the gut. All four *Lactobacillus* species isolated were found to tolerate low pH of 3.04 and bile concentration of 0.3% as presented in Table 2.

Table 2. Physiological characteristic of *Lactobacillus* isolates

Isolates	Low acid tolerance (pH 3.0)	Bile tolerance (0.3%)
S01 (<i>L. fermentum</i>)	+	+
S02 (<i>L. plantarum</i>)	+	+
S06 (<i>L. plantarum</i>)	+	+
S08 (<i>L. fermentum</i>)	+	+

Key: + = isolate tolerates the condition

Antibacterial activity of the *Lactobacillus* species isolated from fermented *P. biglobosa*

The antimicrobial activity of the 4 *Lactobacillus* species isolates against three (3) isolates of *Pseudomonas* spp. and one (1) isolate of *Salmonella* spp. is illustrated in Table 3 and Figure 1. Isolate S06 (*L. plantarum*) had antibacterial activity against all test bacteria with inhibitory zones ranging from 11 mm to 12 mm. Isolates S02 (*L. plantarum*) and S08 (*L. fermentum*) had activity against at least three of the test bacteria with inhibitory zones ranging from 10 to 18 mm and 5 to 20 mm, respectively. Isolate S01 (*L. fermentum*) had zones of inhibition ranging from 6 to 15 mm but no activity against *Salmonella* spp. and one of the *P. aeruginosa* tested.

Table 3. Antibacterial activity of isolates against *Salmonella* spp. and *P. aeruginosa*

Isolates / test organism	Mean zones of inhibition (mm)			
	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	<i>Salmonella</i> spp.	<i>P. aeruginosa</i>
S01 (<i>L. fermentum</i>)	15	6	0	0
S02 (<i>L. plantarum</i>)	18	0	15	10
S06 (<i>L. plantarum</i>)	12	12	11	12
S08 (<i>L. fermentum</i>)	20	5	7	0

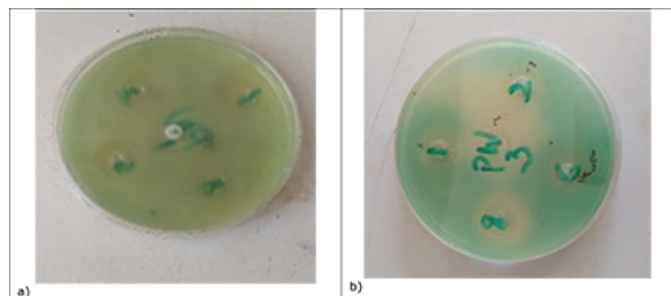


Figure 1. Antibacterial activity of *Lactobacillus* species against: (a) *Salmonella* species and (b) *Pseudomonas aeruginosa*

Discussion

In this study, four *Lactobacillus* species were isolated from locally fermented locust beans. Two isolates were found to be *Lactobacillus fermentum* and the remaining two were *Lactobacillus plantarum*. These species of *Lactobacillus* are among the normal microbiota of fermented foods. *Lactobacillus plantarum* is among the most frequently isolated species of LAB from fermented foods (Xiong et al., 2012; Hutkins, 2019; Szutowska and Gwiazdowska, 2021). Similarly, Stojanovski et al. (2020, 2021) reported that *L. plantarum* was the most commonly isolated *Lactobacillus* species from homemade raw sausages and homemade white sheep's milk cheeses, respectively.

Ability of potential probiotic isolates to tolerate acidic condition and bile salt is commonly used to detect the viability and activity of probiotic organisms. Survival of the isolates at pH 3.0 is considered the optimal acid tolerance for the selected probiotic strains. All the *Lactobacillus* species isolated were able to tolerate pH 3.0, hence they are acid tolerant *Lactobacillus* strains. This result is in line with the report of Azat et al. (2016) who isolated acid tolerant *Lactobacillus* species from traditionally fermented Xinjiang cheese.

All the test *Lactobacillus* species screened in this study were able to tolerate 0.3% of bile salt. This means the isolates can survive in GIT since *Lactobacillus* strains, which grow and metabolize in normal physical bile concentration, could survive in gastrointestinal transit. This is in agreement with the findings of Meira et al. (2012) and Azat et al. (2016).

Bile tolerance is usually considered a basic property of probiotic bacterial strains, as this property enables them to survive in the small intestine. In the human gastrointestinal tract, the mean bile concentration is about 0.3% and is considered crucial and high enough to screen resistant strains (Azat et al., 2016).

Antibacterial activity is an important property of potential probiotic bacteria as this property will inhibit the growth of pathogenic organisms and prevent gastrointestinal infection. The isolates produce various antimicrobial substances which create unfavorable conditions for the growth of pathogens as well as toxigenic and pathogenic organisms in humans and/or animals (van der Meulen et al., 2007).

Results from the antibacterial screening in this study revealed variability in the antibacterial activities of the 4 *Lactobacillus* isolates against *P. aeruginosa* and *Salmonella* spp. The inhibition of growth of the test pathogens by the *Lactobacillus* isolates suggests that they have antimicrobial activity and can inhibit the growth as well as the proliferation of pathogenic bacteria. Susceptibility of *P. aeruginosa* to *Lactobacillus* species observed in this study is similar to the reported by Szutowaska and Gwiazdowska (2021). Inhibitory activity of *Lactobacillus* species against clinical and environmental isolates of *E. coli* was previously reported by Atta et al. (2020).

The variation in inhibitory activity of *Lactobacillus* species observed in this study indicated that the antibacterial activity of *Lactobacillus* species is strain dependent. A similar phenomenon was also observed by Patel et al. (2014), Atta et al. (2020) and Szutowaska and Gwiazdowska (2021).

Lactobacillus plantarum S06 inhibited the growth of the test bacteria in this study. This might be linked to the bacteriocin production ability of *Lactobacillus plantarum* as reported by other researchers (da Silva Sabo et al., 2014; Biswas et al., 2017; Yi et al., 2020; Szutowaska and Gwiazdowska, 2021).

The antibacterial property of the *Lactobacillus* species isolated might be due to the production of metabolites. Metabolites such as lactic acid, acetic acid, diacetyl, fatty acids, aldehydes, bacteriocins, and other compounds, among which, lactic acid, acetic acid, and bacteriocins are the most active antimicrobial compounds produced by *Lactobacillus*

species (Kumari et al., 2012). Production of lactic and acetic acid by LAB lowers the pH of the surrounding thereby inhibiting pathogens and spoilage microorganisms in food especially fermented foods (Reis et al., 2012). These organic acids inhibit the proliferation of bacteria by penetrating the bacterial cell membrane and result in cytoplasmic acidification as well as inhibition of the activities of enzymes (Cervantes-Elizarrarás et al., 2019).

Conclusion

Four isolates of *Lactobacillus* spp. consisting of two *Lactobacillus fermentum* and *Lactobacillus plantarum* were isolated from fermented locust beans seed. All of the four isolates tolerated low pH, 0.3% bile salt and show different degree of inhibitory properties against *Pseudomonas aeruginosa* and *Salmonella* species.

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