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# Isolation of *Lactobacilus Fermentum* as Probiotics for Aquaculture from some Indigenous Fermented Food Products in Nigeria

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**Abstract**

This study aims to evaluate the probiotic and antimicrobial properties of *Lactobacillus fermentum* isolated from some indigenous fermented food products- *Ogi* (fermented corn OG), *Wara* (fermented milk W) and *Fufu* (fermented cassava FU). The isolates were evaluated for their tolerance to low pH, bile salt, antimicrobial potential, tolerance to high temperature and osmotic pressure. The isolates from these fermented products with best antimicrobial properties were identified using PCR amplification and sequencing of 165 rDNA. Results showed that total of 135 isolates were cocci or rod shaped, gram positive, haemolysis negative and catalase negative and these were identified as lactic acid bacteria. Most of the isolates showed strong antibacterial activity to *A. hydrophila*, *E coli*, and Salmonella, however only Fu 9 (*Lactobacillus fermentum* strain F4S8) and OG 1 (*Lactobacillus fermentum* strain BCS27) showed higher zones of inhibition against all the pathogenic organism which is a good sign of a potential probiotics candidate and they were further identified using PCR techniques. Identification by 165 rDNA sequences showed the two isolates were identified as *Lactobacillus fermentum* with Fu 9 identified as *Lactobacillus fermentum* strain F4S8 and OG 1 *Lactobacillus fermentum* strain BCS27 with a similarity index of approximately 99-100%.

**Keywords:** *Lactobacillus fermentum,* fermented food products, Genetic Analyzer, antimicrobial

## Introduction

The fermentative capacity and other beneficial roles of lactic acid bacteria (LAB) has been known for years (Mckay and Boldun, 1990). These live bacteria that have a beneficial effect on the host are referred to as probiotics (Fuller, 1989). Results from various studies had shown that probiotics have the ability to lower pH, produce antimicrobial agents, improve performance of animals, reduce lactose intolerance and improve immune response (TenBrink *et al.,*1994; Jin *et al.,* 1998; Matsuaki *et al.,* 1998). Various studies has shown the effectiveness of probiotics use in aquaculture (Verschuere *et al*.,2000, Moriaty 1998, Gram *et a.,l* 1999, Faramarzi *et al.,* 2011, Ajani *et al.,* 2012). These beneficial bacteria are administered to the fish mainly through feed and water (Verschuere *et al.*, 2000, Austin *et al.*, 1995, Gomez-Gil *et al.*, 1998, Robles *et al*, 1998).

Probiotics has shown in aquaculture the ability to colonize gastrointestinal tracts when administered over time (Balcazar *et al*., 2006). Probiotics has also been used to grow microalgae and rotifers and it showed a better result than the control (Gomez *et al*.,2002) while Saini *et al*., (2014) reported increased performance of *Labeo rohita* fed probiotics supplemented diet.

High probiotics has also shown its ability to improve immune system of fish (Zhou *et al*.,2010, Nwanna *et al*., 2013), nutrient digestion (Bacazal *et al*., 2006) and reproduction (Carnevali *et al*., 2013). The use of probiotics has been encouraged in aquaculture because of its numerous benefits. The isolation of indigenous Lactic Acid Bacteria (LAB) from food products has frequently been reported, including LAB from raw cow milk, camel milk and traditional cheese and dairy products (Khedid *et al*., 2009; Lee *et al*., 2013 and Iranmanesh *et al.,* 2014). However, studies on the properties of LAB isolated from Nigeria's local fermented food product such as *Ogi* (fermented corn), *Wara* (fermented milk) and *Fufu* (fermented cassava) have not been reported. The aim of this study was to isolate the LAB from these fermented food products and identify them by genetic analyzer 3130xl sequencing.

## Materials and Methods

### Isolation, Screening and Identification of Probiotics

Probiotic bacteria were isolated from three fermented products, *Ogi* (fermented corn), *Wara* (fermented milk) and *Fufu* (fermented cassava). 1g each of these fermented foods was serially diluted from dilution $10^{-1}$ to $10^{-10}$ with each test tube containing 9ml of distilled water. Dilution $10^{-1}$, $10^{-3}$ ,$10^{-5}$ ,$10^{-7}$ and $10^{-9}$ was plated by taking 1ml from each test tube into the petri dish and labeled. 70g of de Mann Rogosa Sharpe (MRS) agar was weighed into a clean conical flask and dissolved in 1000ml of distilled water, homogenized and autoclaved for 15min at 121°C. It was allowed to cool and was poured aseptically into the labelled petri dishes, swirled gently, allowed to solidify and incubated anaerobically at 37°C for 24 hrs. From the mixed colonies, a total of 135 lactic acid bacteria were sub-cultured to get a pure culture.

### Gram's staining

This was carried out to know the morphology of the lactic acid bacteria selected and classified to shapes and Gram reaction. Heat fixed smear was prepared from each of the isolates selected. Crystal violet (primary stain) was added to each slide and allowed to stay for 60sec. the slide was washed with tap water. Gram's iodine (mordant) was added to each slide and allowed to stay for 40sec, rinsed with tap water and decolourised using 95% ethanol. The slide was rinsed and counter stained using safranin (secondary stain) and allowed to stay for 60sec, then it was rinsed, air dried and observed under the microscope using x100 objective (Oil immersion objective).

### Catalase test

Catalase test was carried out on each of the isolate. This was used to check the production of enzyme catalase. A drop of 3% $H_2O_2$ was taken and dropped on the microscopic slide aseptically. A loopful of bacterial culture was taken and mixed with the $H_2O_2$ solution on the slide and the presence of the bubble production observed (Goyal *et al*., 2012).

### Haemolysis test

Heamolysis activity of the isolate was investigated by making a smear of LAB on prepared blood ager using 7% sheep blood. The plates were incubated anaerobically at 37°C for 24-48hrs after which they were observed for clear zones around the colonies.

### Antibacterial potentials

The antibacterial properties of the selected LAB were examined by culturing the LAB in MRS broth anaerobically for 24hrs. The broth was centrifuged to obtain cell free supernatant at 5000rmp for 15 minutes. The pathogenic organism used were *Aeromonas hydrophila*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* spp and *Bacillus cerus* isolated from fish and freshwater pond. Each of the bacteria was sub-cultured on a nutrient agar overnight and was suspended in sterile water. A concentration of $10^8$cfu/ml, 0.2ml from each bacterial was spread on a Mueller Hilton ager after which a sterile 5mm diameter cork borer was used to make a hole on the

surface of the ager. Each of the LAB was put into the hole and incubated overnight at 37$^0$C after which the zoon of inhibition was measured (Barefoot and Klaenhamme, 1984).

**Bile Tolerance**

Bile salt tolerance of each LAB was investigated. Each isolate was activated in MRS broth for six hours anaerobically at 37$^0$C. 1ml of each culture was inoculated into MRS supplemented with different concentration of bile salt (0.2,0.4,0.6 and 1%). The growth in the bile salt was monitored by reading the optical density in a spectrophotometer at 650nm after 24hrs of incubation at 37$^0$C.

**pH Tolerance**

The ability of the selected LAB to survive at low pH of 4, 3.5, 3 and 2.5 was checked. MRS broth was prepared and adjusted to different pH ranges and incubated at 37$^0$C for 24hrs.

**Molecular Characterisation**

**Molecular Analysis**

DNA was extracted using the protocol stated by Frank *et al*., (2008). Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28$^0$C. After this period, cultures were centrifuged at 4600x g for 5 min. The resulting pellets were re-suspended in 520 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0).

Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were added. The mixture was incubated for one hour at 37$^0$C, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and mixed. The suspension was incubated for 10 min at 65$^0$C and kept on ice for 15 min.

An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 x g for 20 min. The aqueous phase was transferred to a new tube, isopropanol (1: 0.6) was added and DNA was precipitated at –20 ºC for 16 h.

DNA was collected by centrifugation at 7200 x g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer.

**PCR reaction cocktail**

10 µl of 5x GoTaq colourless reaction, 3 µl of MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and -1525R, 5′-AAGGAGGTGATCCAGCC-3′. primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. PCR carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) Pcr profile and initial denaturation, 94°C for 5 min; 30 cycles, of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds; and a final extension at 72°C for 10 mins. And chill at4$^0$C.GEL (2,3)

**Integrity**

The integrity of the amplified product about 1.5Mb gene fragment was checked on a 1% Agarose gel ran to confirm amplification. This was done by mixing 8µl of amplified product to 4µl of loading dye and ran on the solidified Agarose gel at 110V for about one hour. Picture taken under UV light. Also the amplified product was checked on a nanodrop of model 2000 from thermo scientific to quantify the concentration of the amplified product.

**Purification of Amplified Product**

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added to about 40µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mixed thoroughly by vortexing and kept at -20$^0$C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70% ethanol and mixed then centrifuge for 15 min at 7500 g and 4°C. Again, remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. Then resuspend with 20 µl of sterile distilled water and kept in -20$^o$C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about one hour, to confirm the presence of the purified product.

**Sequencing**

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer (165 rDNA) from Applied Biosystems using manufacturers' manual, while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio-Edit software and MEGA 6 were used for all genetic analysis.

**Results**

**Isolation and Screening of Probiotics**

A total of 135 isolates were cocci or rod shaped, gram positive, haemolysis negative and catalase negative were identified as lactic acid bacteria and were screened for their probiotics quality. For excluding potential pathogens in aquaculture, antagonistic qualities of the LAB is of utmost importance (Huis in't Veld and Shortt, 1996).

**Antibacterial Potentials**

The isolates were challenged against some pathogenic organisms *in-vitro* and varying zones of inhibitions was recorded while the one with the best zones of inhibition were selected for further studies (Table 1). Some of the isolates showed strong antibacterial activity to *A. hydrophila*, *E coli*, Salmonella etc. Only Fu 9 and OG 1 showed higher zones of inhibition in all the pathogenic organism which is a good sign of a potential probiotics candidate. Fu9 and OG1 were selected for further studies on their probiotics potentials.

**Table 1:** Antibacterial potentials

| | ZONE OF INHIBITION IN (mm) | | | | | |
|---|---|---|---|---|---|---|
| LAB | *A. hydrophila.* | *E. coli* | *Staphylococcus* | *Pseudomonas sp* | *Bacillus* | *Salmonella* |
| FU 9 | 12±0.45 | 7±0.53 | 9±1.24 | 14±2.1 | 13±0.43 | 18±2.41 |
| OG 1 | 14±0.79 | 9±0.47 | 7±2.13 | 17±0.75 | 15±1.32 | 16±1.56 |
| WA 3 | 3±0.21 | R | R | 2±1.3 | R | R |
| FU 4 | R | 2±2.31 | R | R | R | R |
| OG 5 | R | R | R | R | 5±2.13 | R |

**Note:** FU—FUFU, OG—OGI, WA—WARA, R- NO RESISTANCE

**Bile Tolerance**

The isolates that showed very good potentials as probiotics was further checked for their bile tolerance. The result showed that the selected isolates survived bile salt concentrations of 0.2 to 1% though the survival was decreasing as the bile salt increases as shown in Table 2.

**Table 2:** Bile tolerance result

| LAB | 0.2% | 0.4% | 0.6% | 1.0% |
|---|---|---|---|---|
| FU 9 | 1.853 | 1.593 | 1.516 | 1.261 |
| OG 1 | 1.801 | 1.562 | 1.427 | 1.248 |

**Acid Tolerance**

The selected isolates demonstrated tolerance to acidic medium from 2.5 to 4. The viability of the isolates increases as the pH increases towards neutral (Table 3).

**Table 3:** Acid tolerance test

| LAB | 2.5 | 3.0 | 3.5 | 4 |
|---|---|---|---|---|
| FU 09 | 1.112 | 1.354 | 1.389 | 1.562 |
| OG 01 | 1.241 | 1.295 | 1.463 | 1.515 |

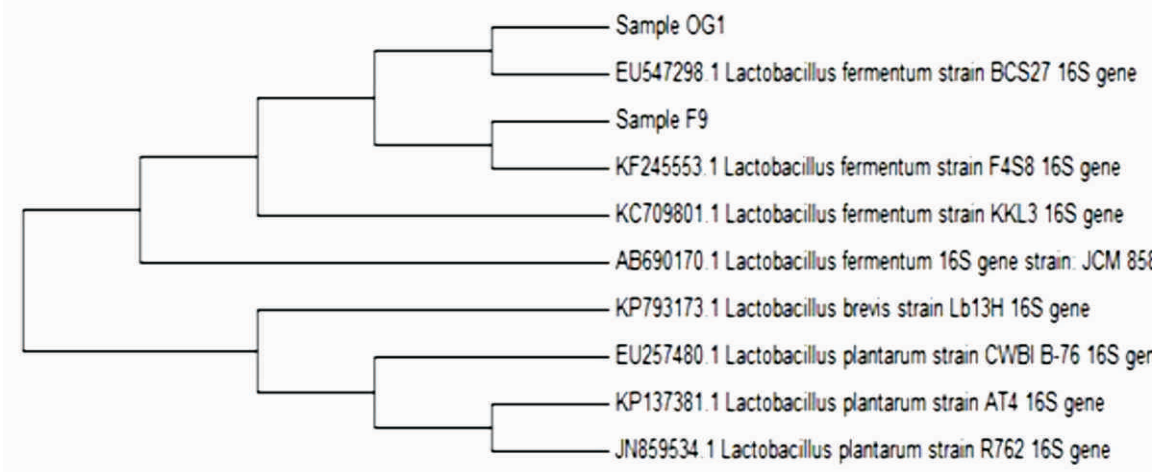


**Figure 1:** Phylogenetic tree showing the identified strains of *L.fermentum*

**Phylogenetic analyses**

The phylogram based on multiple sequence alignment of the 2 bacteria isolates examined here, along with 8GenBank reference sequences is shown in Fig. 1. The main nodes of the tree separated all species with high bootstrap values. Reference sequences of the three species L. *plantarum*, *L. brevis* and *L. fermentum* used in the phylogeny computation were clearly delineated into 2 major phylogenetic clades with the species of *Lactobacillus fermentum* grouped separately from those of *Lactobacillus brevis* and *Lactobacillus plantarum*.

Sample OG1, identified as *Lactobacillus fermentum* despite the SNPs and Indels were closely related to *Lactobacillus fermentum* strain BCS27 and Sample F9 was also identified as Lactobacillus fermentum as it was closely related to *Lactobacillus fermentum* strain F4S8.

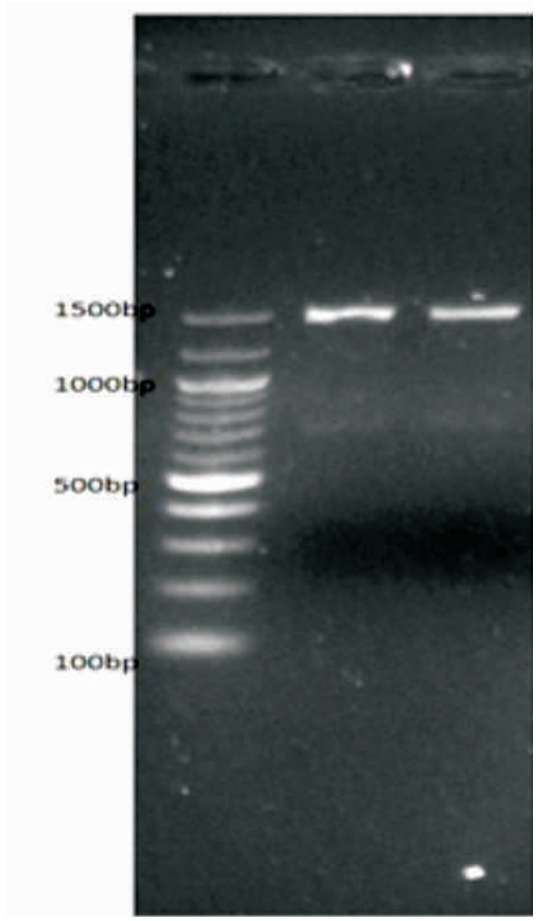


**Figure 2:** PCR gel integrity of bacteria isolates using 16SDNA universal primers

## Discussions

A total of 135 isolates were cocci or rod shaped, gram positive, haemolysis negative and catalase negative were identified as lactic acid bacteria and were screened for their probiotics quality. For excluding potential pathogens in aquaculture, antagonistic qualities of the LAB are of utmost importance (Huis in't Veld and Shortt, 1996). In this study some of the isolates showed strong antibacterial activity to *A. hydrophila*, *E coli*, Salmonella etc. Only Fu 9 (*Lactobacillus fermentum* strain F4S8) and OG 1 (*Lactobacillus fermentum* strain BCS27) showed higher zones of inhibition against all the pathogenic organism which is a good sign of a potential probiotics candidate. This agreed with the findings of

Nwanna *et al*., (2013) and Srinu *et al*., (2013) which evaluated different lactic acids bacterial strains for probiotics characteristics and found them resistance to *E. coli*, *Pseudomonas aeruginosa*, *Staphyllococus aureus* and *Salmonella paratyphi* and recommended them as functional probiotics. This antibacterial activity may be due to the production of substances like bacterocins as suggested by (Karimi Torshizi *et al*., 2008; Sihag and Sharma 2012). The finding in this study also agrees with that of Al-allaf *et al*.,(2009) who studied antibacterial activity of isolates from minced beef meat and found them very active in inhibiting *E coli*, *salmonella* and *staphyllococus. L. fermentum* can only provide the needed health benefits in fish if it can overcome the bile and acid barriers in the intestinal tract (Gibson *et al.,* 2000). Bile tolerance are very important characteristics of a potential probiotics. Bile is required for bacteria growth in the intestine (Lee and Salminen, 1995). The bacteria isolates from this study showed very high tolerance to bile salt which made them very good probiotics candidate. This result is similar to the report of Srinu *et al*., (2013) and Barakat *et al*., (2011) which evaluated different lactic acid bacteria strains for probiotics and found them tolerant to bile salt. Karimi Torshizi *et al*., (2008) recorded a high level of bile tolerant by *L. fermentum* TMU121 in probiotics screening for poultry. It has been recorded that one of the selection criteria for probiotics is tolerance in acidic medium (Karimi Torshizi *et al*., 2008; Gibson *et al*.,2000). The selected isolates in this study were exposed to pH ranging from 2.5 to 4 and recorded increase in survival as the pH increases. The LAB showed a high survival at pH 2.5 which is a good quality for a potential probiotic. Karimi torshizi *et al*., (2008) recorded the same trend as observed in this study. Dadir (2012) recorded survival of *L. acidophilus* and *B. lactis* at pH 2 which supports the findings of this study. Usman (1999), recommended a survival at pH of 3 for 2h and 1,000mg/l bile concentration as optimal acid and bile concentration for probiotics.

In conclusion, it can be said that strains of *L. fermentum* strain F4S8 and *L fermentum* strain BCS27 isolated from locally available fermented cassava and corn showed great potentials as a probiotics candidate for use in aquaculture. They showed high survival in acid and bile medium which indicates that they can survive in large quantity after passing the digestive tracks and the stomach. Their antagonistic properties also points to their ability to inhibit the growth of pathogenic organisms in the gut of the fish and hence play a major role in the health of the organism.

**References**

Ajani, E. K, Nwanna L. C. and Ikwuemesi J. C. (2012). Effects of *Lactobacillus acidophilus* on Growth and Immune Responses of African catfish, *Clarias gariepinus.* Proceedings of the 6th World Fisheries Congress, May 7-11, Edinburgh, UK.

Austin, B., Stuckey L.F., Robertson P.A.W, Effendi I. and Griffith, D.R.W. (1995). A probiobic strain of vibro alginolyticus effective in reducing disease caused by *Aeromonas salmonicida, vibrio anguillarm* and *vibrio ordalii. Journal of fish diseases* 18: 93-96.

Al-Allaf M .A .H, Al-Rawi A .M .M. and Al-Mola A. T. (2009). Antimicrobial activity of lactic acid bacteria isolated from minced beef meat against some pathogenic bacteria. *Iraqi Journal of Veterinary Science* 23:115-117.

Balcazar J.L, de Blas I, Ruiz-Zarzuela .I, Cunningham D, Vendrell D and Muzquiz J.L. (2006). The role of probiotics in aquaculture. *Vetinarary Microbiol*;114:173-186.

Barakat, O.S., Ibrahim, G., Tawfik, N., El-Kholy, W. and Gad El-Rab, A. (2011). Identification and probiotic characteristics of *Lactobacillus* strains isolated from traditional Domiati cheese. International Journal of Microbiology Research 3: 59-66.

Barefoot. S. and Klaenhamme T. R. (1984). Detection and activity of lactacin B, a bacteriocin produced by *lactobacillus acidophilus. Applied and Environmental Microbiology*, 45; 1808-1815.

Carnevali O. Avella M A and Gioacchini .G. (2013). Effects of probiotics administration on zebrafish development and reproduction. *General and comparative endocrinology*. 188:297-302.

Faramarzi M. Kiaalvandi S and Farnaz .I (2011). The effect of probiotics on growth and body composition of common carp (Cyprinus carpio). Journal of animal and veterinary advances

10(16): 2408-2413.

Frank J.A. Reich, C. I. Sharma S, Weisbaum, J . S, Wilson, B.A and Olsen G.J. (2008). Critical Evaluation of Two Primers Commonly Used for Amplificationof Bacterial 16S rRNAGenes. *Applied and Environmental Microbiology,* 74: 2461–2470.

Fuller, R,, (1989). Probiotics: The scientific Basis. Chapman and Hall, London, New York.

Gram, L., Melchiorsen J,, Spanggaard B,, Huber I and Nielsen T. (1999). Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* strain Ah2.

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