

ISOLATION AND ANTIBIOGRAM OF PROBIOTIC LACTIC ACID BACTERIA FROM  
TIGERNUT MILK IN AWKA, NIGERIA

NWOZOR, N.C\*., IKELE, M.O., NWIYI, I.U. OGUNO, S.I. & OSILO, C

\*Department of Applied Microbiology and Brewing,  
Nnamdi Azikiwe University, Awka, Nigeria

**ABSTRACT**

Since viable probiotic bacteria as dietary supplements have attracted a great deal of research interest, Different sources of food especially fresh agricultural produce such as nuts and vegetables have become excellent reliable sources of probiotics from Lactic acid bacteria (LAB). In this research, Tigernut milk samples, produced from an agricultural root crop were used for the isolation of probiotic Lactic acid bacteria. Among the samples, four isolates were identified as: *Lactobacillus* sp, *Streptococcus* sp, *Lactococcus* sp and *Pediococcus* sp based on their morphological and biochemical characteristics. The Isolates were resistant to NaCl (2.0% to 4.4%), exhibited luxuriant growth at low pH (in the acidic condition), while maximum growth was observed at pH around pH 4.4 The isolates were examined for their antibacterial activity against six different test pathogens, which resulted to the effective inhibition of all test pathogens, with maximum inhibitory activity against *Staphylococcus aureus* (26.1 mm) and minimum was against *Pseudomonas aeruginosa* (8 mm). The results of the present research strongly suggest that, Tigernut milk obtained from Awka metropolis is a great source of probiotic Lactic acid bacteria. Extensive research on the isolation and optimization of Lactic acid bacteria from Tigernut milk is required for development of probiotic enriched food supplements for our great benefit.

**Keywords:** Lactic Acid Bacteria, Probiotics, Tigernut

**INTRODUCTION**

In recent years, increased awareness of the health and well-being of people globally have necessitated the partial switch from animal-based food products to natural and health foods with nutrient balanced profile required for various metabolic, physiological and other functional demands (Cavera *et al.*, 2015). Plant foods are very essential to man's health and survival due to the presence of protein and carbohydrate of about 70% and 80% respectively.

Tiger nut (*Cyperus esculentus*) is an underutilized crop which belongs to the division-*Magnoliophyta*, *Classiliopsidea*, order-*Cyperales* and family-*Cyperaceae* (family). The nut can be eaten raw, roasted, dried or baked. It is a good source of starch, glucose, protein and minerals such as potassium, phosphorus, vitamin E and C. The tiger nut milk is very nutritive and it serves as a source of energy for all categories of people. Its fat contains a large amount of oleic acid which is heart friendly and helps to prevent cardiac arrest. It also aids the internal mechanisms and prevents both constipation and diarrhoea. (Asante *et al.*, 2014)

The Lactic Acid Bacteria (LAB) have been isolated and identified as probiotics from many sources in nature, including human milk, dry-cured meat products, and some Nigerian cereal-based-fermented food such like *ogi* (fermented cereal gruel) (Bamishaiye *et al.*, 2011). For Lactic Acid Bacteria to be considered probiotics, they must display antibacterial activity, must be proven safe for the consumer, exhibit tolerance to bile and gastric acid (Tamang *et al.*, 2016).

The use of probiotics with broad antagonistic potential and natural antimicrobial substances is considered as an appropriate alternative to antibiotic treatment and a better pharmaceutical approach (Sadeghi *et al.*, 2022). This research is aimed at the isolation and probiotic characterization lactic acid bacteria from tigernut milk sold in Awka, Nigeria.

## **Methodology**

### **Sample Collection**

Forty samples of tigernut milk sample were obtained from different vendors at four different vending areas in Awka Metropolis as Eke Awka Market: Unizik Junction, Aroma and Ifite. The tigernut milk samples were collected using sterile containers and transported to the Microbiology laboratory, Nnamdi Azikiwe University for bacteriological analysis.

### **Isolation of lactic acid bacteria**

LAB were isolated on MRS agar supplemented with bromocresol purple as an indicator and incubated at 37 °C for 24–48 hours. Single bacterial colonies that produced a yellow zone were selected according to morphological differences. Gram-staining and catalase tests were carried out. Only Gram-positive and catalase negative colonies were sub-cultured in MRS broth and kept in glycerol before experimental use.

### **Antibiotic sensitivity test**

The isolates were inoculated into a nutrient broth and incubated at 37°C for 24 hours. The isolates (0.1 ml) were inoculated using the spread-plate technique on Mueller-hinton agar plates using sterile swab glass spreader. Antibiotic discs, containing antibiotics were placed on the top of the agar plates and incubated at 37°C overnight. The isolates were categorized as susceptible or resistant to antibiotics according to the presence/absence of zones of clearing around the antibiotic discs.

### **Tolerance to low acid (pH) conditions and NaCl**

Acid tolerance was determined by comparing the absorbance after 3 hours with the initial absorbance at 0 hour spectrophotometrically at wavelength of 600 nm; uninoculated sterile MRS broth served as the blank. Similarly, for the NaCl tolerance, viability of cells in MRS broth supplemented with 2%, 4% and 6.5% (w/v) NaCl after incubation at 37°C for 24 hours indicated a positive result.

MRS broth (10 ml) was dispensed into test tubes and adjusted to pH values of with HCl. The test tubes were inoculated with 0.1 ml of overnight MRS broth culture of the Lactic acid bacteria cultures and inoculated microaerophilically at 37°C for 3 hours. The absorbance values of the cultures were checked, before and after 3 hours of incubation,

### **Antimicrobial activity**

Antagonistic potential of Lactic acid bacteria cultures were evaluated against serious food borne as well as spoilage causing microorganisms. Overall antimicrobial activity of cell free supernatant was checked against pathogens: *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* using well diffusion method. The wells were inoculated with 150 µL of 24 h old culture supernatant and the plates, which were inoculated with the test pathogens were incubated at 35°C for 24 h. The antibacterial activity was determined and zones of inhibition were measured in millimeter (mm).

## **Results**

### **Identification of the LAB Isolates based on Morphology and Biochemical Characterization**

Lactic acid bacteria were isolated on MRS agar at 37°C under anaerobic conditions. The lactic acid bacterial isolates obtained were confirmed as rods and cocci respectively by microscopic examination (Table 1).

All the isolates were Gram positive, catalase negative, unable to utilize citrate and no urease and indole production was observed by any of the isolates. On the basis of morphological and biochemical characteristics, isolates were tentatively identified at genus level as *Lactobacillus* sp, *Streptococcus* sp, *Lactococcus* sp and *Pediococcus* sp,

Out of the four Lactic acid bacteria isolates, *Pediococcus* sp gave clear zones around the lawn of indicator bacterial strains using bit/disc method with broadest antimicrobial spectrum. The largest diameter of inhibition (up to 26.1 mm) was obtained against *Staphylococcus aureus* by the inhibitory activity of *Lactococcus* sp (Figure 1)

**Tolerance to low pH**

In the present investigation, the LAB isolates were subjected to acid tolerance at a low pH range of 2.5 to 4.4 with a high levels of pH tolerance, which was indicated by its absorbance readings at 600nm for a duration of 3hours (Figure 2). The ability of the LAB isolates obtained in this research to survive gastric pH (even as low as 2.5) makes it a choice organism for probiotic studies.

**Antimicrobial activity of Lactic Acid Bacteria Isolates**

The antagonistic potential of the Lactic acid bacteria isolates were tested against test food borne pathogens. Fig 1 describes the inhibitory spectrum of the LAB against test pathogens. All the LAB isolates exhibited a broad spectrum of inhibition against test pathogens with zones of inhibition ranging from 8.0 mm-26.1 mm.

**Antibiogram of Lactic acid Bacteria Isolates**

The selected isolates were assessed for their antibiotic susceptibility against different antibiotics. *Lactobacillus* sp was found to be resistant to most of the antibiotics, while *Streptococcus* sp exhibited high sensitivity towards most of the test antibiotics.

**Table 1: Identification of the LAB Isolates based on Morphology and Biochemical Characterization**

TEST	<i>Lactobacillus</i> sp	<i>Streptococcus</i> sp	<i>Lactococcus</i> sp	<i>Pediococcus</i> sp
Gram Reaction	+ Rods	+ Cocci	+ Cocci	+ Cocci
Catalase	-	-	-	-
Motility	-	-	-	-
CO <sub>2</sub> from Glu	+	-	+	-
Carbohy Ferment Glucose	+	+	+	+
Lactose	+	+	+	+
Xylose	+	-	+	+
Sucrose	+	+	-	+
Mellibiose	-	-	-	-
Raffinose	+	-	-	+
Sorbitol	-	-	-	+
Growth in NaCl 2%	+	+	+	+
4%	-	+	+	+
6.5%	-	-	-	-

**Key:** += Positive Result

-= Negative Result

**Table 2: Antibiogram of LAB isolates**

ORGANISM	ANTIBIOTICS									
	PEF	CPX	S	CEP	NA	CN	AU	PN	SXT	CXF
<i>Lactobacillus</i> sp	+	+	-	-	-	+	+	-	-	-
<i>Streptococcus</i> sp	+	-	-	+	-	-	+	+	-	+
<i>Pediococcus</i> sp	+	-	-	+	-	+	-	-	+	-
<i>Lactococcus</i> sp	+	-	+	-	-	-	+	-	-	+

**Key:** += Sensitive

- = Resistant

**PEF**-Pelfoxacin

**CN**-Cephalexin

**CEP:** Cefepime

**NA:**Nafacin

**CPX**-Ciprofloxacin

**AU**-Augumentin **SXT:**Trimethoprim-Sulfamethoxazole

**S**-Streptomycin

**PN:**Penicillin

**CXF:**Cefoxitin

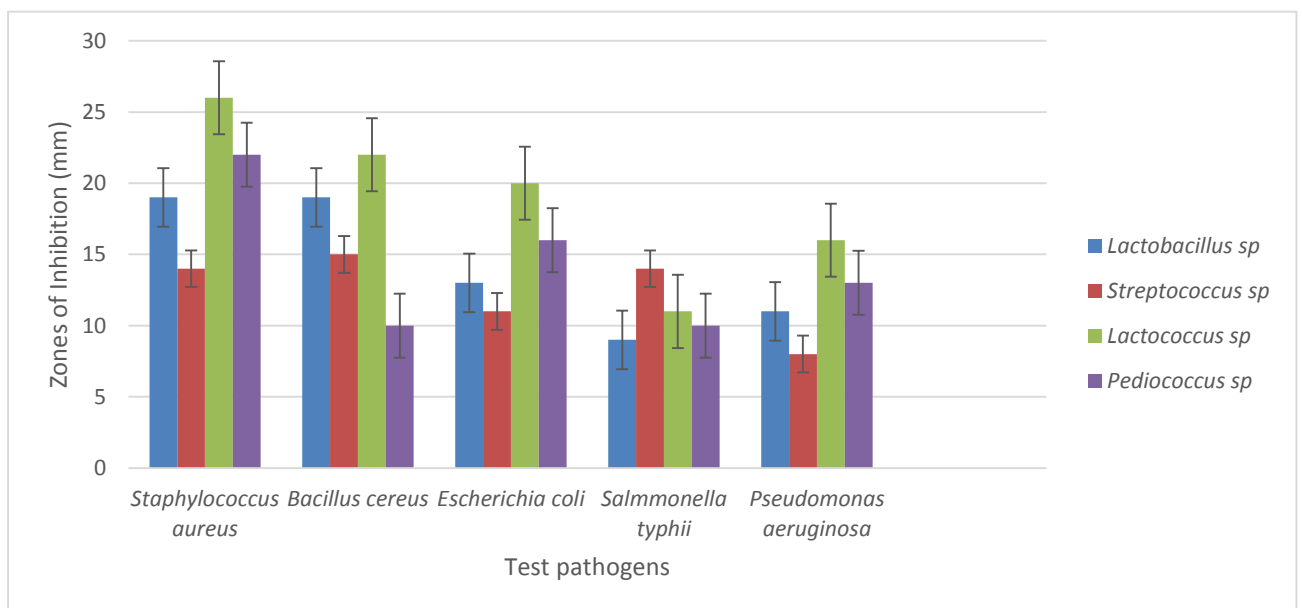


Fig 1: Antimicrobial activity of Lactic Acid Bacteria isolates

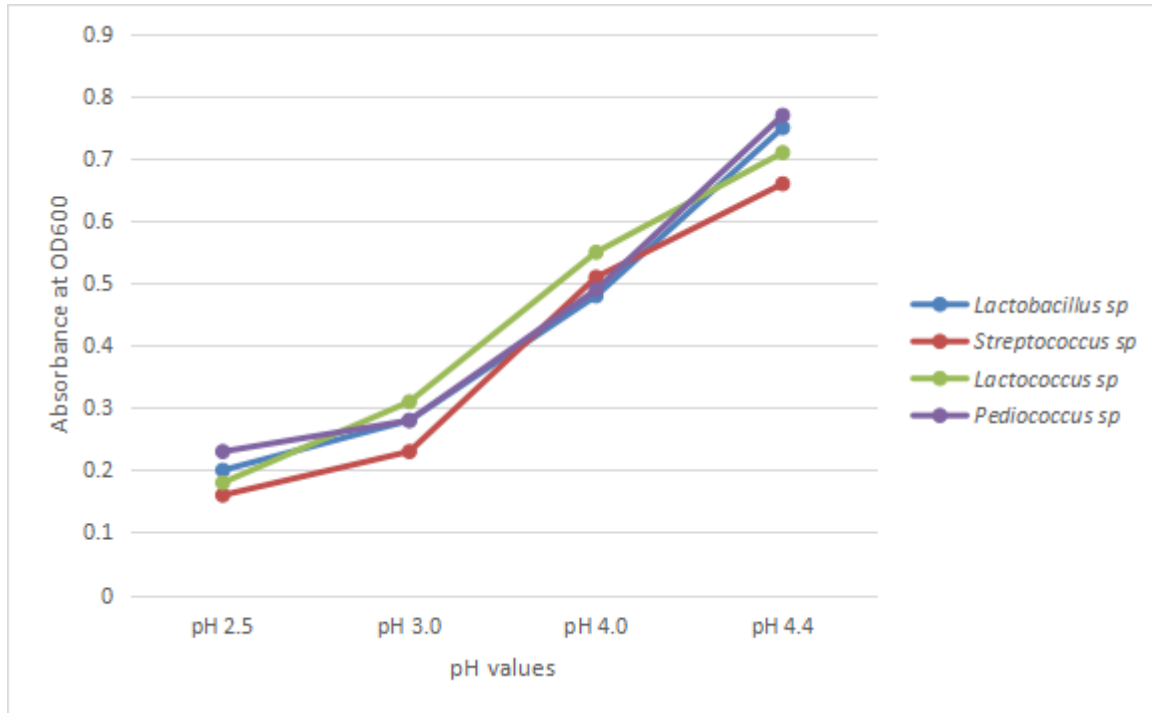


Fig 2: pH tolerance of Lactic Acid Bacteria Isolates

## DISCUSSION

A total of 40 LAB isolates were obtained from different samples of tigernut milk. In the first step, macro- and microscopic evaluation, Gram staining, and catalase-producing ability were used to confirm that the isolated bacteria belong to the group of LAB. Both rods and cocci were found to belong to Gram-positive bacteria, and all were marked as catalase-negative.

The cell morphology of all isolates was evaluated through microscopic observation and the majorities (N=31) were found to be cocci and the remaining (N=9) were rod shaped. By comparing the result of morphological, physiological and biochemical tests the isolates were grouped in to four genera of lactic acid bacteria. With this, isolates were grouped in to *Lactobacillus sp*, *Lactococcus sp*, *Streptococcus sp* and *Pediococcus sp*. The number of isolates grouped in the identified genera of LAB showed in (Table 1). Similar reports showing the production of lactic acid bacteria from fresh food or drink from agricultural produce have been documented (Rodriguez *et al.*, 2021). Lactic acid bacteria (LAB) belong to a group of gram positive bacteria that produce lactic acid as their main fermentation product into the culture medium and generally recognized as safe (Konings, 2000).

There are several criteria for bacteria to recognize as probiotic. One of the most important features is tolerance to low pH concentrations. They must overcome physical and chemical barriers in the gastrointestinal tract to be able to transit through the stomach. The antimicrobial properties of probiotics are attributed to their metabolite productions including organic acids, hydrogen peroxide, and bacteriocin (kouhi

*et al.*, 2022). The production of metabolites, such as organic acids by LAB can inhibit Gram-negative bacteria through the penetration of cell membrane and therefore affecting its functioning, acidifying cytoplasm and inhibiting acid-sensitive enzymes (Cervantes-Elizarrarás *et al.* 2019).

In this study, the LAB isolates were investigated against Gram-positive and Gram-negative pathogens. The evaluated isolates showed better antibacterial effects on Gram-positive than Gram-negative indicators, which this could be due to the outer membrane of Gram-negative bacteria. *Lactococcus* sp and *Pediococcus* sp showed the best results and were able to inhibit the growth of all the pathogen indicators. The antimicrobial properties of starter cultures are one of the most essential functional features necessary for determining the probiotic characteristics of the isolated strains (Szutowska & Gwiazdowska, 2020).

Due to the possibility of antibiotic resistance transfer from food microorganisms like LAB to human pathogens, a basic study was performed to determine the obtained strains' resistance towards commonly used antibiotics. In this regard, all isolates of LAB were screened against a broad spectrum of antibiotics that acted as inhibitors of protein synthesis or cell wall synthesis. Detailed results regarding the susceptibility of the isolated probiotic strains to the most common antibiotics are presented in Table 2.

Based on the antibiotic susceptibility assay, all isolates were sensitive to PEF and STP, they were however resistant to NA, CN and PN. These findings are consistent with those from other studies (Colombo *et al.* 2020), which also reported that LAB isolated from different environments, like fermented olives, curly kale, or dairy products, were resistant to Streptomycin, and other antibiotics. Bacteria of the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc* have been frequently reported to possess strong natural resistance to the above-mentioned antibiotics (Colombo *et al.* 2020). Several LAB species belonged to especially *Lactobacillus*, *Pediococcus* and *Leuconostoc* have been reported to exhibit high levels of natural resistance to streptomycin, which is useful to separate them from other Gram-positive bacteria (Michalak *et al.* 2018).

### **Conclusion**

The study was conducted to isolate and identify the naturally occurring lactic acid bacteria from fresh tigernut milk obtained from different vendors in Awka metropolis. The lactic acid bacteria isolates were identified as belonging to the genus: *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Pediococcus*. The LAB isolates relatively inhibited the growth of the test pathogens. Moreover, antibiotic susceptibility was satisfactory and the resistance profile was characteristic for the LAB group. The results demonstrate that there is a diversity of lactic acid bacteria with great probiotic properties in tigernut milk. Further research on the in-vivo studies of probiotic potential of the lactic acid bacteria isolates is highly recommended.

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