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Original Research Article

Screening of Probiotic Characteristics of Lactic Acid Bacteria Isolated from Some Fermented Nigerian Food Products

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ABSTRACT

Microorganisms that exert health benefits to the host when consumed in the right proportion are termed Probiotics. Certain lactic acid bacteria, especially *Lactobacillus spp.* have been considered to be probiotics with proven health benefits and have been used widely in human health management. In this study, *in vitro* and *in vivo* evaluation of probiotics characteristics of Lactic acid bacteria (LAB) isolated from traditional Nigerian fermented food products (Ogi and Kunu-zaki) was carried out. Ethical approval was obtained from Covenant Health Research Ethics Committee (CHREC). Ogi and Kunu-zaki were plated on de Mann Rogosa (MRS) agar, and *Lactobacillus spp.* were isolated and identified by morphology and biochemical characteristics. The probiotic characteristics such as acid and bile salt tolerance, antibiotic susceptibility and haemolytic activity were performed. A total of 15 lactic acid bacteria was isolated. Only 4 (26.7%) of the isolates have high tolerance at pH values 2, 4, and 6.5 for 6 h and hence were reported. The four acid-tolerant isolates showed higher survival rates to 0.1 and 0.3% bile salt for 3 h. All bile and acid tolerant isolates showed different sensitivity to various antibiotics used. Two out of the four probiotics were found to survive the gastrointestinal tract of male albino Wistar rats when they were given a daily dose of $1x10^9$ cfu/mL for two weeks. The four probable *Lactobacillus spp.* were found to possess characteristics of probiotics and could have the ability to confer health benefits.

Keywords: Probiotics, Acid tolerant, Bile salt-tolerant, Antibiotic sensitivity.

Introduction

Lactic acid bacteria (LAB) are Gram-positive rod/cocci, aerotolerant, acid-tolerant, usually nonsporulating, and non-respiring group of microbes occupying a major part in the traditional process of food fermentation. They help in the preservation of food products by impeding the growth of spoilage/pathogenic microbes such as bacteria and also enhance the organoleptic attributes of foods.[1, 2] Some lactic acid bacteria are confirmed to be probiotics, while others are likely to be potential probiotics. Some of these include *Lactobacillus acidophilus, L. rhamnosus, L. reuteri, L. Pontis, L. plantarum* and *L. pentosus*. Recently, probiotics had gained a wide usage due to the health benefits associated with them. Some of these health benefits include their use in the treatment of diarrhoea, irritable bowel disease and cancer among others.[3] Traditional fermentation of cereals is a vital food improvement practice in Nigeria and most developing countries, most importantly, due to the nutritional value, economic importance, preservation of foods in places where means of safe storage such as refrigeration facilities are unavailable. Ogi is one of the most common indigenous health-promoting foods in the western part of Nigeria. It is predominantly produced from fermented maize (*Zea mays*), sorghum (*Sorghum vulgare*), or millet (*Pennisetum americanum*) gruel.[4] Ogi is used as weaning foods for infants in the western region of Nigeria and also consumed by adults. Raw consumption of Ogi has been reported to reduce pains and discomforts from people suffering from gastroenteritis in some communities in the southwest of Nigeria.[5,6] Ogi is fermented by LAB, and this has been frequently isolated from it.[7] More work has been done on the nutritional benefits present in Ogi and the roles of the diversity of fermenters present.[7-,9] The addition of other ingredients such as wheat offal to ogi help to further fortify it and reduced malnutrition problems among infants.[9] Kunu-zaki is a popular non-alcoholic Nigerian beverage made from grains. This drink is very common among the Northerners of Nigeria. It is consumed generally by more than 70% of this populace on a regular basis.[10] Various kinds of Kunu are being produced, and their names are usually based on the substrate used in the production, organoleptic attributes, locality, or any extra added ingredient during production. For instance, Kunu gyada has groundnut seed paste added, Kunu tsamiya have tamarind fruit pulp added, Kunu bururu have fermented cow milk added, and Kunu zaki has sugar added without the use of any preservative. The most important grain used in the preparation of kunu-zaki is millet. Millet is one of the most popular, nutritious grains and has numerous health benefits to human well-being, thus making kunu a very nutritional drink.

This study aims to isolate and characterize probiotic LAB from Ogi and Kunu-zaki.

Materials and Methods

Ethical approval and experimental animals

Covenant Health Research Ethics Committee (CHREC) approved this study with an approved protocol assigned number CHREC/48/2020. Twenty-one 8 weeks old male albino Wistar rats were purchased,

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housed in plastic cages, and allowed for acclimatization for one week. After which the rats were assigned to three groups: Group T1 (Group treated with isolate M1), T2 (Group treated with isolate M4), NT (Control).

Sample collection

Freshly prepared Ogi was obtained from the Iju market in Ota, Ogun State, and freshly prepared kunu-zaki from Covenant University Cafeteria, Km 10, Idi-iroko road, Canaan land, Ota, Ogun state respectively.

Isolation of lactic acid bacteria

For isolation of LAB, 1 mL kunu-zaki and 1g of ogi were added to the distinct 9 mL of sterile distilled water, respectively. Subsequently, a serial dilution of the isolates was prepared up to the dilution six. An aliquot (0.1 mL) of the 3rd and 6th dilution were spread plated on de Mann, Rogosa, and Sharp (MRS) agar (HiMedia) plates, which was anaerobically incubated with the application of (Gas Pak Anaerobic Systems, BBL) for 48 h at 37°C. After that, single colonies were selected at random from MRS plates and streaked on MRS agar to obtain pure colonies. The purified isolates were characterised by morphology, Gram's reaction and biochemical tests using Bergey's Manual of Systematic Bacteriology.

In vitro probiotic property screening of LAB isolates

Tolerance to acidity of LAB isolates

The identified LAB isolates were evaluated for growth in an acidic environment. MRS broth was acidified with HCl, and the pH was adapted to pH 2.0 and pH 4.0, respectively. Acidified (pH 2.0 and pH 4.0) and non-acidified broth (pH 6.5) which is the control were inoculated with lactic acid bacteria isolates and anaerobically incubated at 37 °C for 6 h. The optical density of the cells was determined using Spectrophotometer at 600nm after incubation[11].

Bile tolerance test of LAB isolate

The rate of survival of LAB at varying concentrations of bile salt was investigated. The LAB was cultivated in MRS broth containing 0.1% and 0.3% bile salt, which was further subjected to incubation at 37°C (anaerobic environment) for 3 h.[12]

The following equation was used to calculate the survival percentage of the test organisms after 3 h.

$$\text{Survival\%} = \frac{Log\ N1}{Log\ N0} \times 100$$

Where;

LogN1 is the logarithm of absorbance values of cultures at 0.1 and 0.3% bile salts. LogN0 is the logarithm of absorbance values of cultures at 0% bile salt.

Antibiotic susceptibility test of LAB isolates

Each of the isolates that have tolerance to bile and acid was selected for susceptibility test to antibiotics. This test was carried out by Kirby-Bauer disk diffusion technique.[13] Antibiotics used include; ciprofloxacin (5µg), erythromycin, co-trimoxazole (15µg), chloramphenicol (10 µg), penicillin (10µg), tetracycline (30µg), neomycin (10µg), and gentamicin (5µg). 100 µL of freshly prepared isolates of acid and bile-tolerant LAB was plated on Mueller-Hilton agar using a sterile cotton swab. Discs impregnated with antibiotics were carefully set on the surface of the agar, and the antibiotic discs were allowed to diffuse through the agar for 30 min at 4°C and incubated at 37°C for 24h with CO_2. Resistance, Intermediate and Susceptible results were determined by the zone of inhibition.

Growth of LAB isolates at varying temperatures

A 50 µL of freshly prepared LAB isolates was measured and inoculated into four different tubes containing 5ml of modified MRS broth medium, A drop of 0.12 g/L concentration of bromocresol purple was added to the medium which serves as an indicator. Two separate tubes containing inoculated LAB was incubated at 15°C and 45°C respectively for 24 h. Survival at different temperature was observed after the incubation period, and colour change of the broth from purple to yellow also indicates that the organism was able to survive and grow at different temperatures.[14]

Tolerance to different NaCl concentrations

The tolerance of the LAB isolates to varying NaCl concentrations was investigated so, 4% and 6.5% of the salt were considered for evaluation. Additionally, test tubes containing 5 mL of MRS broth that have been modified by adding bromocresol purple were adjusted accordingly to the desired level of NaCl composition. Four distinct test tubes containing 4% and 6.5% NaCl each were inoculated individually with 50 µL of 1 per cent of 24 h old LAB culture and subjected to 7 days of incubation at 37°C. Colour change was observed, and purple to yellow colour change was presented as evidence showing the growth of cells.[14]

Haemolysis

Haemolytic activities of LAB isolates were done. The LAB isolates were inoculated on blood agar plate via the streaking technique, and incubation was carried out anaerobically for 48 h at 37°C. Afterwards, the culture plates were observed for Alpha-haemolysis, Beta-haemolysis and Gamma haemolytic activities. Isolates with no haemolytic activities were chosen for further screenings.[15]

Probiotic administration and total faecal LAB count

The acclimatized rats were assigned to three groups: T1 (Group treated with LAB M1), T2 (Group treated with LAB M2), Group T3 (Control, without LAB isolate treatment). Oral inoculation solution of *Lactobacillus spp.* was prepared to contain a final concentration of $1x10^9$ lactobacilli/ml and given to the rats for two weeks after which faecal samples from each rat was collected the day of sacrifice and analysed using MRS agar for the enumeration of total faecal LAB count.

Statistical analysis

Statistical analysis was conducted with a one-way analysis of variance (ANOVA) and post hoc Tukey's comparison test using Version 5.01 of GraphPad Prism software. Data were expressed as mean ±SEM values.

Results and Discussion

The research aimed at studying various probiotic activities of microorganisms had gained so much interest recently because of their wide application in human health therapy and the bio-preservation of food products. As a result of this, LAB present in locally fermented Nigerian food products (Ogi, Kunu-zaki) have been isolated, screened, and selected based on safety and functional foods properties to obtain LAB with probiotic activities.

The characteristics (morphological, biochemical, and physiological) of the four selected potential probiotic bacteria showed them to be LAB and presumptively identified to be *Lactobacillus spp*. Precisely, three of them are homofermentative and one heterofermentative *Lactobacillus spp.* (Table 1). This is in concord with the LAB isolated from Ergo (An Ethiopian traditionally fermented product).[14]

The strains of LAB considered in this study grew well at 37 °C after incubating for 24 h, which is the optimum temperature for their growth. There was a reduction in the growth of these isolates at 15 °C and 45°C (Table 2).

The result showed all the isolates grew well at 4.0% and 6.5% salt NaCl concentration (Table 2). Lactic Acid Bacteria isolated from various sources like milk produce, fermented cereals and meats are able to survive 1.0 to 0.9% NaCl concentration.[16] This trait showed by these potential probiotics LAB could be relevant in the food industries and for preservation.

Following FAO guidelines on safety evaluation of probiotics, the selected strains must be safe in the host. The selected strains should not have haemolytic activity as this shows they are not virulent. All the strain considered showed no haemolytic activity and were selected

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for other tests since their safety was confirmed (Table 2). Similar studies show that most of the LAB strains show no haemolytic activity.[17]

In this study, only 4 (26.7%) out of the 15 LAB isolates examined for survival at pH 2, 4, and 6.5 showed higher survival rate ranging from 7.7% to 25% at pH 2 and 15.4% to 95.0% at pH 4 (Figure 1). Only the four isolates were reported in this study. This result is in accordance with a previous study that indicated that there is a high reduction in the population of LAB isolates when grown in strong acidic condition[18]. Although, these results are in contradiction with other reports obtained from other studies, which showed that most isolated strains of *Lactobacillus spp.* from different sources have a survival rate of 38.40% to 73.29% and above 80% at pH 2 respectively.[16,19]

Another essential selection criterion for probiotic isolates is bile salt tolerance, and this shows the organisms have a high tolerance and can adapt to the bile concentration in the small intestine. The four acid-tolerant lactic acid bacteria isolates exhibit high tolerance to bile salt conditions 0.1% bile salt (78.5%-97%) and 0.3% bile salt (77.0%-92.1%) thereby making them good probiotic organisms (Figure 2).

Assessing antibiotics susceptibility patterns is an essential criterion in the selection of prospective probiotics. Probiotic organisms to be selected should not serve as reservoirs in the transport of antibiotic-resistance genes to pathogens present in intestines. All of the isolates were found to be resistant to co-trimoxazole antibiotics. Isolate M1 was found to be resistant to all the tested antibiotics.

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Table 1: Characteristics (Physiological, morphological, and biochemical) of the LAB isolates

		Morphology		Sugar Fermentation			
Isolate	**Gram's reaction**	**Shape**	**Catalase**	**Sucrose**	**Glucose**	**Lactose**	**Urease**
M1	+	Rod	-	**+ G**	**+G**	+	-
M2	+	Slender rod	-	+	+	+	-
M3	+	Slender rod	-	+	+	+	+
M4	+	Slender rod	-	+	+	+	-

KEY: Positive (+), Negative (-), Gas production (G)

Table 2: Haemolytic activity, growth at different temperatures and NaCl concentration of isolates

	Salt Concentration		Growth at different Temperature		
LAB Isolates	**4% NaCl**	**6.5% NaCl**	**15^{o}C**	**45^{o}C**	**Haemolytic activity**
M1	+	+	+	+	No haemolysis
M2	+	+	+	+	No haemolysis
M3	+	+	+	+	No haemolysis
M4	+	+	+	+	No haemolysis

KEY: Positive (+), Negative (-), Gas production (G)

Table 3: Antibiotic susceptibility test of isolates

ISOLATES	**Gentamicin**	**Ciprofloxacin**	**Chloramphenicol**	**Penicillin**	**Neomycin**	**Co-trimoxazole**	**Tetracycline**	**Erythromycin**
M1	R	R	R	R	R	R	R	R
M2	S	R	S	I	S	R	I	S
M3	S	S	S	S	I	R	I	S
M4	I	R	R	S	I	R	R	R

KEY**:** Resistance (R), Susceptible (S), Intermediate (I)

Table 4: Total faecal lactobacilli count of the tested LAB isolates on MRS agar

Test Isolate	**10^{-5} cfu/mL**	**10^{-6} cfu/mL**
T1(M1 Treated)	2.2×10^{7}	4.0×10^{7}
T2(M4 Treated)	1.8×10^{7}	1.0×10^{7}
NT(Control)	2.9×10^{6}	2.0×10^{6}

Two of the isolates were found to be resistant to erythromycin and chloramphenicol and two susceptible to the same antibiotics (Table 3). Lactobacillus M1 and M4 showed the best probiotic potential and were chosen for *in-vivo* studies to check their survival in the GIT of rats. The two chosen species survived the gastrointestinal tract of rats which is evidenced by the total viable faecal LAB count (Table 4). This result helps for further confirmation of the bile and acid tolerance ability of these isolates in the *in-vitro* study.

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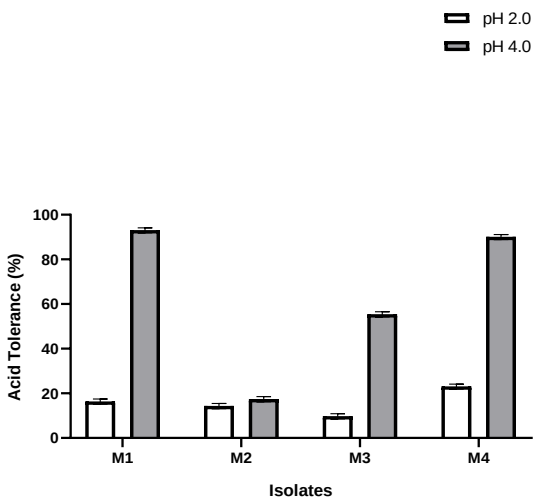

Figure 1: Survival rates of isolates at pH 2.0 and pH 4.0

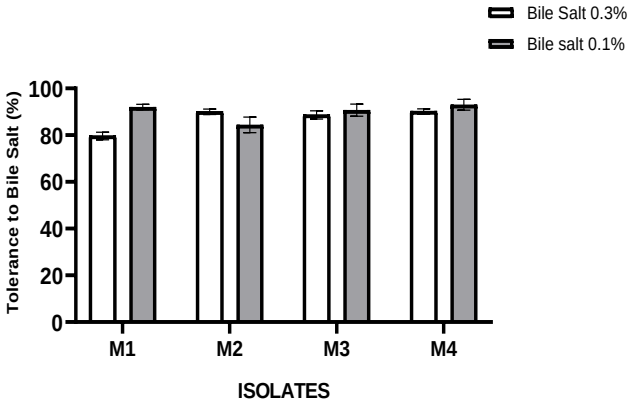

Figure 2: survival rates of isolates at 0.1% and 0.3% bile salt

Conclusion

In this study, four lactic acid bacteria isolates from ogi and kunu-zaki, which were identified presumptively as *Lactobacillus spp.* were shown to possess probiotic properties and characteristics. This shows that ogi and kunu-zaki are good carriers of probiotics. It is therefore recommended that their frequent consumption should be encouraged.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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