Probiotics Potential of Bacteria Associated with Fermented Unripe Plantain (*Musa paradisiaca*) Juice Sold in Orieugba Market Umuahia, Nigeria

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Abstract: Probiotics are live microorganisms that when administered in adequate amounts, confer a health benefit on the host. They are naturally present in fermented foods, may be added to other food products and are available as dietary supplement. This study was aimed to determine probiotics potentials of bacteria associated with fermented unripe plantain (*Musa paradisiaca*) juice. The physico-chemical analysis of the fermented unripe plantain (*Musa paradisiaca*) juice was determined by homogenizing 10g of the samples in 20ml of distilled water and using a referenced glass electrode pH meter. Total titratable acidity was also determined by titrating 0.1N sodium hydroxide against 10 ml of supernatant of homogenized sample, using phenolphthalein indicator. The result showed that the pH was 5.72, total suspended solid was 620mg/l and total titrable acidity was 0.35ml**.** Total bacteria counts from fermented unripe plantain (*Musa paradisiaca*) juice indicated that the most appropriate fermentation time that yield highest probiotic organisms was at the 21th hour (8.4 × 10^3 cfu/ml), followed by 24th hour (7.2 ×10^3 cfu/ml) while the least (1.5 × 10^3 cfu/ml) was recorded at 48th hour. A total of four probiotic bacterial species were isolated and identified using molecular techniques, which includes; *Leuconostoc pseudomesenteroides, Weissella confusa, Gluconobacter frateurii* and *Weissella cibaria. Gluconobacter frateurii* was the only Gram negative organism isolated from the juice while the other three organisms were all Gram positives. Evaluation of probiotic properties of the isolates showed that the four isolates had maximum growth at temperature of 30^{o}C. *Leuconostoc pseudomesenteriodes, Weissella cibaria and Weissella confusa* showed tolerance to 6.5% NaCl. The current study demonstrated that ulcer eradication was significantly higher when lower dose (2 ml/kg) of the fermented unripe plantain juice was administered with percentage severity of 69.17% while it was non-significant when a higher dose of (4 ml/kg) was administered with percentage severity of 115.71%. It is evident from the present study that consumption of fermented unripe plantain (*Musa paradisiaca*) juice exerts health benefits to the consumers.

Key word: Probiotics, Fermentation, Gastrointestinal, Techniques, Molecular

INTRODUCTION

Probiotics are live microorganisms that when administered in adequate amounts, confer a health benefit on the host (Hill, *et al*., 2014). Utilization of beneficial microorganisms in health promotion is not new, and in fact they have been consumed by humans, especially in the form of fermented dairy foods, for many years (Ranadheera *et al*., 2010). The most common genera that have been used and possess probiotic characteristics are the lactic acid bacteria; *Bifidobacterium* and *Lactobacillus*. Probiotic bacteria are beneficials in lowering serum cholesterol levels and reduction of risk factors for colon cancer by nutritional enrichment and metabolic effects. (Parvez, *et al*., 2016).

Food fermentation represents one of the oldest known uses of biotechnology (Eduardo, *et al.*, 2016). Fermented foods and beverages form a significant proportion of all diets worldwide; they are typically about one-third of the foods consumed globally (Eduardo, *et al.*, 2016). Fermentation of food covers a wide range of microbial and enzymatic processing and is used to achieve desirable characteristics such as prolonged shelf-life, improved safety, attractive flavour, nutritional enrichment and promotion of health (Machado *et al*., 2014). Traditional fermented foods are generally fermented by lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, *Lactobacillus pentosus, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus. casei, Leuconostoc mesenteroides, Lactobacillus. kimchi, Lactobacillus fallax, Weissella confusa, Weissella koreenis* and *Pediococcus pentosaceus*, which are

considered as the probiotic source of the food practice. Availability of certain specific nutrients such as vitamins, minerals, and acidic nature of fruits and vegetables provides conducible medium for fermentation by LAB.

Plantain (*Musa paradisiaca*) is known to serve dual purpose of both dietary and therapeutic roles (Ajijolakewu., *et al*., 2021), and have been used in folk medicine for the treatment of both infectious and non-infectious diseases and for wound healing (Shodehinde and Oboh, 2013; Pereira and Maraschin, 2015; Amutha and Selvakumari, 2016). Various researchers have reported on the ethnomedicinal, antimicrobial and phytochemical properperties of *Musa paradisiaca* including anti-ulcer potential of the fermented unripe *Musa paradisiacal* in wistar rats (Ikpeazu, 2017). Consumption of fermented plantain juice has a propensity to affect human health and mental wellbeing positively by stimulating the activities of the gut microflora through the production of bioactive chemicals and neuropetides (Holzapfel, *et al*., 1998). However, to the best of our knowledge, the bacteria associated with the fermented unripe plantain juice has not been fully evaluated and reported. This paper reports the probiotic potential of bacteria associated with fermented unripe plantain juice and their identification at the molecular level.

MATERIALS AND METHODS

Sample Collection: The (plantains) samples used for this study were purchased from Orie ugba market in Umuahia North L.G.A, Abia State. The samples were then transferred to Linnc laboratory Umuariaga Junction Umudike, Abia State in the morning, the same day it was collected for analysis.

Physicochemical analysis of the sample:

pH: The pH was determined by homogenizing 10 g of the various samples in 20 ml of distilled water and using a referenced glass electrode pH meter (JENWAY 3020, England).

Titratable acidity (TA% lactic acid): This was determined by titrating 0.1N sodium hydroxide against 10 ml of supernatant of homogenized sample, using phenolphthalein indicator as described by AOAC (1990).

Total Suspended solid (TSS): The sample was filtered through a pre-weighed filter paper, then the filter paper was dried in an oven to remove remaining water and weighed again. The weight difference over the sample volume provides the total suspended solid concentration in Mg/l

Sample Preparation: The unripe plantain were peeled with a sterile knife, washed with sterile water and sliced into pieces with the aid of a sterile sharp knife. The samples were put in a sterile container which hermetically closed with a top. All containers were placed at room temperature that was set at 28°C and allow to ferment for forty eight (48) hours (Akoa *et al*., 2014).

Isolation of Lactic Acid Bacteria from fermented plantain juice: Media used in this study was De Mann, Rogosa and Sharpe's (MRS) agar. It was prepared according to manufacturer's instruction by weighing 67.1 g of MRS powder which was dissolved in 1litre of distilled water and sterilized by autoclaving at 121^{o}C for 15 minutes at 15 Psi. It was allowed to cool before pouring into sterile petri dishes. The isolation of Lactic Acid bacteria (LAB) were done after 6, 9, 12, 15, 18, 24 – 48 h to determine the time LAB had the highest colony forming unit (CFU) in the juice. For LAB isolation, 0.1 ml of each of the fermented plantain juice were inoculated into already prepared MRS agar plates (Using spread plate technique) and incubated at 37°C for 24-48 h for bacterial growth. The plates were observed for appearance of colonies and number of colonies produced on each plate was recorded. Bacteria were purified by streak plate method on MRS agar and incubated at 37°C for 24 h to obtain a pure culture. The obtained pure cultures were transferred into MRS agar slants and then maintained in refrigerator at 4°C (Yuanyan, *et al*., 2017).

Evaluation of Probiotic Potentials: The probiotic properties of the isolates were evaluated by checking the pH tolerance, temperature sensitivity and sodium chloride (NaCl) tolerance. The pH tolerance was done by inoculating the isolated bacterial cultures into sterile MRS broth tubes of varying pH (4.0, 6.0 and 8.0) and incubated at 37°C for 2 days. Then 0.1mL inoculums from each tube was transferred to MRS agar medium by pour plate method and incubated at 37°C for 48h. To determine temperature sensitivity and pH, the selected bacterial culture were grown at different temperature (15°C, 30°C, 45°C) and varying pH (4.0, 6.0, 8.0) for 48-72h. Then 0.1ml inoculums was transferred to MRS plates by pour plate method and incubated at 37°C for 48h. Salt tolerance of selected bacterial cultures were also assessed after 2days of incubation at 37°C using concentration of 1.0, 3.0 and 6.5% NaCl in MRS broth (Yuanyan, *et al*., 2017).

***Induction of Ulcers in Rats*:** Gastric ulceration was induced in the rats according to the procedure described by Sayanti *et al* (2007). A total of twenty albino rats were deprived of food for 24 hours and grouped into four groups (Group I, II, III and IV) of five rats each. Group I severed as normal control which received only distilled water and every animal in Groups II- IV received 100mg/kg indomethacin orally after 30 minutes of treatment. After one hour, all the rats were sacrified by cervical dislocation. Their abdomens were removed and stomachs excised after which the ulcer scores were determined.

Macroscopic Ulcer Index (U.I): The severity of the ulceration was graded according to the method of Kunchandy *et al.,* 1985. Ulcer scores and descriptive remarks are as follows;

0 = Normal gray colour stomach, 1 = Vascular congestions, 2 = Number of ulcers less than 5, 3 = Number of ulcers more than 5, 4 = Ulcer with bleeding and 5 =Perforation of duodenal wall.

The ulcer index was calculated by summing the entire number of ulcers plus the severity of the ulcer.

Identification of The Isolates: Genomic DNA was extracted from isolated bacterial cultures (Green and Sambrook, 2012), and PCR amplification of 16S rDNA gene was achieved using universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG - 3') and 338R (3'- TCTCAAACTAGACCGAGTC - 5'). The polymerase chain reaction was carried out using thermal cycler. Denaturation of double stranded DNA (dsDNA) temperate was done at 92^0C while annealing of primers at 55^0C and then extension of dsDNA molecules at 72^0C. Various chemical components used for PCR include, $MgCl_2$, buffer (pH 8.5), Deoxynucleotide triphosphate (dNTPs), Universal primer and thermostable DNA polymerase. The consensus sequence of the 16S rDNA gene was generated from forward and reverse sequence data using aligner software and EMBOSS merger. A BLAST search was performed with the 16s ribosomal RNA sequence database to find the closest homologous sequence.

Statistical Analysis: The results were analyzed using one-way analysis of variance (one-way ANOVA) by using the statistical package for social sciences (SPSS Inc, Chicago, USA) program version 17.0. Turkey's test was used to determine the significant differences between the variables at $p < 0.05$.

RESULTS

The physicochemical analysis of the fermented unripe plantain (*Musa paradisiaca*) juice showed that the pH was 5.72, total suspended solid was 620mg/l and total titrable acidity was 0.35g/l as shown in table 1. The cellular morphology of the LAB isolates that all the isolates were rod shaped. They were all raised and had round colonies as indicated in table 2. Total bacteria counts from fermented unripe plantain (*Musa paradisiaca*) juice indicated that the most appropriate fermentation time that yield highest probiotic organisms was at the 21

hours (8.4×10^3 CFU/mL), followed by 24 hours (7.2×10^3 CFU/mL) while the least (1.5×10^3 CFU/mL) was recorded at 48 hours as show in figure 1. Biochemical tests and Molecular identification showed that the identified organisms are *Leuconostoc pseudomesenteroides, Weissella confusa, Gluconobacter frateurii* and *Weissella cibaria*. Molecular identification of all the isolates were done by PCR using universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG - 3') and 338R (3'- TCTCAAACTAGACCGAGTC - 5'). Four (4) different bacterial species were identified as *Leuconostoc pseudomsenteroides, Weissella confuse, Gluconobacter frateurii* and *Weissella cibaria* with percentage identities of 97.78%, 87.97%, 82.92% and 93.35% respectively as shown in table 4 below. The probiotic properties of the isolates showed that the highest growth of all isolates was at temperature of 30^0C, while *Leuconostoc pseudomsenteroides* had the highest growth at 6.5 NaCl concentration the as shown in table 5 below.

The effect of fermented plantain juice on indomethacin induced ulcer revealed that ulcer eradication was significantly higher when lower dose (2 ml/kg) of the fermented unripe plantain juice was administered with percentage severity of 69.17% while it was non-significant when a higher dose of (4 ml/kg) was administered with percentage severity of 115.71%. Turkey's test using statistical package for social sciences (SPSS Inc, Chicago, USA) program version 17.0 were used to determine the significant differences between the variables at $p < 0.05$.

Table 1: Physicochemical Characteristics of the Fermented Unripe Plantain (*Musa paradisiaca*) Juice

Parameters	Values from Fermented Plantain Juice
Values from Fermented Plantain Juice	5.72
Total suspended solid	620 mg/l
Total titrable acidity	0.35 g/l

Table 2: Morphological Identification of Lactic Acid Bacteria (LAB) Isolates from Fermented Unripe Plantain (*Musa paradisiaca*) Juice

Sample Code	Cultural Morphology	Cellular Morphology
PLO1	White, raised, opaque, round, shiny colonies	rods, purple, chains
PLO2	White, raised, round, shiny colonies	rods, chains, purple
PLO3	Flat, Shiny, cream, raised, round colonies	rods, pairs, pink
PLO4	Shiny, white, raised, round colonies	rods, chains, purple

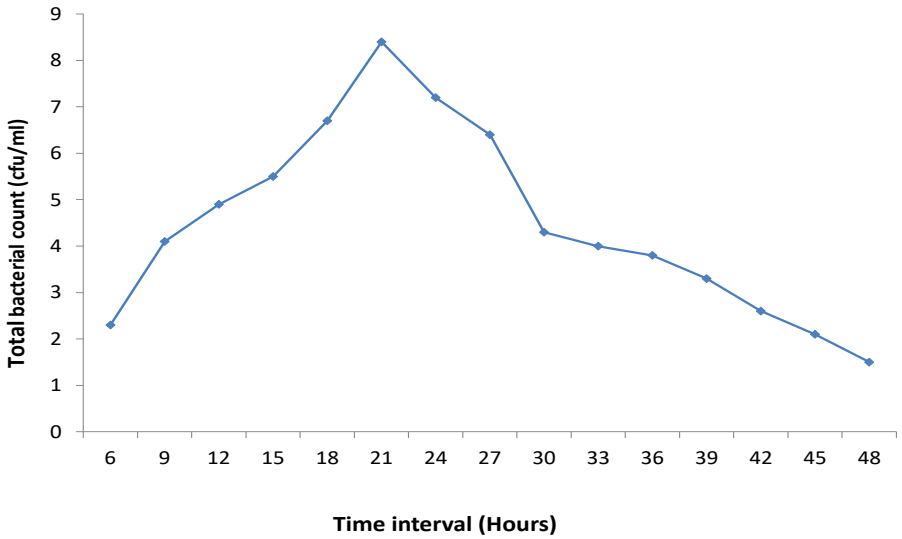

Fig 1: Most appropriate fermentation time that yield highest probiotic organisms

Table 3: Biochemical Tests of the Isolates from Fermented Unripe Plantain (*Musa paradisiaca*) Juice

Sample Codes	GR	CAT	URE	CIT	OXI	LAC	GLU	Probable Organisms
PLO1	+	−	−	−	−	+	+	*Leuconostoc* spp.
PLO2	+	−	−	+	−	+	+	*Weissella* spp.
PLO3	−	−	−	−	−	+	+	*Gluconobacter* spp
PLO4	+	−	−	−	−	+	+	*Weissella* spp

Key: Positive = +, Negative = −, GR = Gram reaction, Cat = Catalase, Ure = Urease, Cit = Citrate, Lac= Lactose, Glu= Glucose

Table 4: Molecular Identification of the Isolates from Fermented Unripe Plantain (*Musa paradisiaca*) Juice

Organisms	E-value	Percentage Identity	Accession No
Leuconostoc pseudomsenteroides	0.0	97.78%	LC519558.1
Weissella confusa	0.0	87.97%	KU324931.1
Gluconobacter frateurii	0.0	82.92%	AB436558.1
Weissella cibaria	0.0	93.35%	MK402189.1

Table 5: Evaluation of Probiotic Properties of the LAB Isolates

Organisms	Temperature			pH			NaCl(%)		
	15^0C	30^0C	45^0C	4.0	6.0	8.0	1.0	3.0	6.5
Leuconostoc pseudomesenteroides	+	++	+	+	+	+	+	+	++
Weissella confusa	+	++	+	+	+	+	–	+	+
Gluconobacter frateurii	+	++	–	+	+	+	+	+	–
Weissella cibaria	+	++	+	+	+	+	–	+	+

Key: No growth = –, Scanty growth = +, Moderate growth = + +

Table 6: Effects of Fermented Plantain Juice on Indomethacin Induced Ulcer

Treatment	Ulcer No	Ulcer Score	Ulcer Index	Severity (%)
Distilled water 5ml/kg	17.25 ± 5.59^{ab}	23.00 ± 6.65^{a}	14.00 ± 1.23^{a}	100
Misoprostol 50 µg/kg	3.25 ± 1.11^{c}	4.00 ± 1.78^{b}	8.73 ± 0.29^{b}	62.36
Juice 2 ml/kg	6.25 ± 1.11^{bc}	10.50 ± 2.25^{b}	9.68 ± 0.31^{b}	69.14
Juice 4 ml/kg	24.20 ± 5.63^{a}	37.80 ± 9.58^{a}	$16.20 \pm 1.51_{a}$	115.71

Key: $^{A, b, c}$ superscript are significant vertically at $p < 0.05$

DISCUSSION

A total of four (4) Lactic acid bacteria were isolated from the fermented unripe plantain juice. These organisms include *Leuconostoc pseudomesenteriodes, Gluconobacter frateurii, Weisella confuse and Weisella cibaria*. Similar isolates were obtained in the work of Yi-sheng *et al.,* 2012, when he fermented *jiang-gua* (fermented cucumbers), a traditional fermented food in Taiwan.

The most appropriate fermentation time that yielded the highest probiotic organism occured after 21 hours (8.4×10^3cfu/ml) while the least was after 48 hours (1.5×10^3 CFU/mL). This study also showed that probiotics have positive effect on the eradication of *Helicbacter pylori* infection which could regulate the immune system and influence metabolic reactions. The most commonly used probiotics that may increase the resistance of gastric barrier and as a result inhibit the growth of some pathogens and their adherence to gastric epithelium (Du *et al.,* 2002).

Evaluation of probiotic properties of the isolates showed that the four isolates had maximum growth at temperature of 30^{o}C. *Luconostoc mesenteriodes, Weissella cibaria and Weissella confusa* showed tolerance to 6.5% NaCl. Growth kinetics of *W. confusa* and *W. cibaria* at varying concentration of NaCl has been reported by Lee *et al.* (2012), where most of the strains were able to withstand 6.5% of NaCl concentration, but failed to grow at 8 % NaCl or above.

The current study demonstrated that ulcer eradication was significantly higher when lower dose (2 ml/kg) of the fermented unripe plantain juice was administered and this was in line with the work of Ikpeazu *et al.*, 2017 which showed that the gastroprotective activity of the extract at different doses and that of the reference drug was statistically significant ($p<0.05$). The higher ulcer score (37.80 ± 9.58^{a}) when 4ml/kg fermented unripe plantain juice was administered probably occurred due to higher alcohol concentration in 4ml/kg fermented unripe plantain juice compared to that of the 2 ml/kg of fermented unripe plantain juice. This is in agreement with the work of El-Maraghy *et al*., 2015 who found out that administration of ethanol in rats causes gastric necrotic damage and subsequent inflammatory cell infiltration and reduces the secretion of bicarbonate, gastric mucus and nitric oxide.

CONCLUSION

It is evident from the present study that consumption of fermented unripe plantain (*Musa paradisiaca*) juice exerts health benefits to the consumers. There is currently declining in the use of conventional drugs in the management of peptic ulcer and promising results have been seen with the use of medicinal plant.

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