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Bio-preservative effect of lactic acid bacteria isolated from fermented cow milk on *nunu*

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Abstract: Purpose: The role of lactic acid bacteria (LAB) in food fermentation cannot be over emphasized. LAB being generally regarded as safe (GRAS) have certain functional properties that enhance its suitability as bio-preservatives. During fermentation of foods they are capable liberating certain metabolites that may be inhibitory or lethal to other microorganisms in the fermenting medium. In this regards, this study focused on the functional properties of LAB as bio-preservative for nunu production. **Method**: The lactic acid bacteria used in this work were isolated from fermented cow milk and were screened for their inhibitory effect, out of seven identified; **Result**: only four LAB strains were capable of this effect (*Lactobacillus pentosus*, *L. plantarum, L. fermentum and L. plantarum*B3). *L. pentosus* had the highest inhibitory zone which ranged from 11 mm to 12 mm on *Bacillus cereus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, while the least inhibitory zone was produced by *L. fermentum*. *L. pentosus* acidified fermented cow milk within 18 hr of fermentation reducing acidity from 0-3.86 than other strains of LAB isolates. The proteolytic screening of LAB isolates revealed that *L. pentosus* had the highest zone (18 mm) while *L. fermentum* had the least proteolysis (12 mm) with no lipolysis. **Conclusion**: Nunu fermented with mixed culture of *L. plantarum* B3 and *L. pentosus* as well as single starter culture of *L pentosus* had better shelf life and kept for 5 days before spoilage occurred relative to 3 days observed for spontaneously fermented nunu.

Keywords: Lactic acid bacteria, spoilage, pathogenic bacteria, nunu, fermenting medium

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Introduction

Nunu (Yogurt-like drink) is a locally fermented dairy product well known to be consumed by some West African countries such as Ghana, Burkina Faso and Nigeria precisely Northern Nigerians [1]. Unlike other fermented dairy products, which can be produced from Camel, goat, Horse, nunu is an opaque white to creamy colour liquid food drink produced only from cow milk [2]. Nunu is a nutritious food similar to yogurt whose intake covers Saharan tribes of West African sub region and extends to the inhabitants of Mediterranean region as well as the Middle East. In Nigeria some tribes calls it nunu while in the Middle East of West Africa, it is known as ''Dahii'' or ''Lassi'' [2]. Raw milk is the most used substrates on which microorganisms that are used for obtaining useful cultures for food and feed industry thrive. It is rich in; nutrients, has a favourable pH, contains air, with a low salt concentration and a low osmotic pressure [3, 4]. These attributes, makes milk to be an extremely suitable medium for a great number of microorganisms and considerable number of species growing in milk [3, 4].

Nunu has low shelf life and it is easily spoilt by spoilage microbes, particularly moulds causing loss of nutritional benefits derived from it. Nutritional analysis of nunu reveals that it contains essential amino acids, calcium, phosphorus, vitamin A, C E, B complex but poor in iron and ascorbic acids like other milk products [5]. In Nigeria, the Normadic Hausa/Fulani cattle herdsmen, who controlled more than 80% of Nigerian cattle rearing [9], predominantly prepares nunu. The consumption of nunu was initially restricted to Fulani/Hausa indigenes while the non-indigenes see the preparation as unhygienic, besides its shelf life being poor but due to its nutritional value and quality control awareness, it is now gaining a wide range of acceptability for intake [9].

Lactic acid bacteria generally regarded as safe (GRAS) belong to the phylum firmicutes and being anaerobic microorganisms are capable of fermenting food substrates to produce diverse metabolic products that serve as potential preservatives for perishable foods. The major LAB genera *Lactobacillus, Leuconostocs, Pediococcus,* and *Streptococcus* have a long history of being safe for use in processing of fermented food. The antimicrobial effect of LAB and its safety for use as preservatives is widely accepted [10-12]. This preservative effect is majorly due to the production of lactic acids and other organic acids during fermentation resulting into the reduction of pH of the fermenting medium [13]. Preservation effect is also achieved by the production of other compounds that are antimicrobials and these include hydrogen peroxides, carbondioxide, diacetyl, acetaldehyde [14-16]. Presently, there is no information on the extension of shelf life of *nunu* using lactic acid bacteria cells as preservatives. However research has been conducted on the use of lactic acid bacteria as food preservatives. Lactic acid bacteria cells from fermented cow milk should be developed into preservatives that could be used to preserve *nunu* of good quality and acceptability.

Materials and Methods

Nunu samples were purchased at three different locations: Mokola round about, Ojoo and Bodija markets area of Ibadan metropolis in sterile sample bottles from Fulani hawkers and transported

to the laboratory in ice pack for microbiological analysis. Samples were analysed at the laboratory, Department of Microbiology, University of Ibadan within hour of sample collection.

Isolation of Lactic acid bacteria (LAB) from nunu samples

Nunu samples were serially diluted, 1 ml of 10 fold serial dilution of each sample was inoculated, and using pour plated method on De Man Rogosa and Sharpe (MRS) agar. The MRS agar plates were incubated for 48 h at 30^{o}C micro-aerophilically using candle jar and sub-culturing of the isolates were made until pure cultures were obtained. Pure isolates were maintained on agar slant for further characterization and identification [17].

Phenotypic Characterization of LAB isolates

The isolates were characterized for presumptive identification based on colony morphology, cell morphology and biochemical tests. The biochemical tests include Gram staining, catalase test, casein hydrolysis to mention but a few. All isolates were tested for sugar fermentation with respect to acid production from D- arabinose, D-xylose, galactose, D-fructose, Lactose, D-raffinose, Mannitol and Sorbitol, Sucrose, Ribose Trehalose, Adonitol, Inulin [18] and their identities were confirmed with Bergey's manual of Systematic Bacteriology [19].

Antibacterial activity of the isolated lactic acid bacteria

The indicators of food borne pathogens and spoilage microorganisms (Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Salmonella typhi) used for testing antibacterial activity were obtained from the Department of Microbiology, University of Ibadan. Test microorganisms were inoculated into nutrient broth and incubated at 37^{o}C for 24 h.

Preparation of cell-free supernatant (CFS)

Each LAB isolate was inoculated in 10 ml of MRS broth and incubated at 30^{o}C for 48 h. After incubation, a cell-free supernatant was obtained by centrifuging the bacterial culture at 6000 ×g for 15 min after which it was filtered through cellulose acetate paper of 0.2 µm to remove the residual cells [20].

Screening of LAB isolates for Antimicrobial Activities

The agar-well diffusion method was used in the screening of LAB for antimicrobial activity. Indicator lawns were prepared by inoculating 20 ml of Mueller Hilton molten agar media with 100 μl (approximately, 10^7 cfu/ml) of an overnight-incubated culture of each indicator organism and allowing them to solidify in a Petri-dish. Wells were bored into the agar with a sterile 5 mm diameter cork-borer and were sealed with two drops of sterile agar. Fifty microliters (50 μl) of the filtered cell-free supernatant of the test LAB isolates were separately placed into the wells. The plates were prepared in duplicates and then incubated at 37^{o}C for 24 h. The plates were observed for possible clearing zones (inhibition zones). The antimicrobial activity was determined by measuring the diameter of the inhibition zones around the well using venier calliper [20].

Determination of the rate of acidification of the lactic acid bacteria isolates

The selected lactic acid bacteria isolates (*Lactobacillus pentosus*, *Lactobacillus fermentum*, *Lactobacillus plantarum* B3 and *Lactobacillus plantarum*, were inoculated each as 1% overnight culture into MRS broth whose pH was adjusted to 6.75 before sterilizing in an autoclave at 121^{o}C for 15 mins. The pH of the broth was taken after autoclaving to be 6.54 before each isolates were inoculated into it and after inoculation; the pH of each LAB isolates was also taken. The inoculated broth culture of each LAB isolates was anaerobically- incubated at 30^{o}C. Acid production was determined by measuring the pH of the medium after 6, 12, 18 and 24 h of incubation [21].

Preparation of skimmed milk agar

Skim milk agar was prepared by weighing 25 g of skim milk and reconstituting it with 250 ml of distilled water. The mixture was stirred thoroughly and autoclaved at 110°C for 10 min. Likewise, 500 ml of 2.5% agar solution was sterilized at 121°C for 15 min. The skim milk and agar-agar solutions were held in a water bath at 50°C for cooling and then the skim milk was poured into the agar bottle and mixed thoroughly. The skim milk agar was poured quickly into plates [22]

Measurement of proteolytic activity

To detect protein hydrolysis, the selected LAB were inoculated on skim milk agar plates and were incubated micro-aerophilically at 30°C for 48 h in a candle jar. The isolated colonies Proteolytic activity were carried out in triplicates and zones of clearance surrounding the isolates were measured and all results were averaged and reported as diameter in mm. LAB that show good proteolytic activity (PA $\geq$ 6 mm) were used for further studies [22].

Screening of LAB isolates for lipolytic activity

Lipolytic activity were carried out on the selected isolates and were grown overnight at 37^{o}C in MRS broth. A loopful of young culture of selected isolates was placed on tributyrin agar plates were incubated at 37^{o}C for 5 days and observation were made daily for halo-formation around the colonies. The radius of the halo-formation (mm) at the end ofincubation period was measured and recorded [20].

Safety analysis of selected LAB isolates

Safety tests such as haemolysis, gelatinase and DNAase were carried out on the LAB isolated from fermented milk.

Procedure for DNAse test

DNAse agar was prepared and Methyl green indicator was added and sterilized at 121^{o}C. The agar was cooled and poured on plates. Isolates were inoculated on the DNAse agar and incubation was made at 37 °C for 48 h. Isolates of LAB without halos-formation or clear zone formations around the colonies were selected for further studies [26].

Procedure for β-haemolysin test

Production of β Haemolysin activity was determined on Blood Agar (Oxoid) containing 5% defibrinised horse blood after 48 h of incubation at 37 °C. Zones of clearing around colonies indicated β-haemolysin production. Isolates without clearance around the colonies were selected for further studies [26].

Procedure for Gelatinase test

Nutrient gelatin was prepared according manufacturer's instruction in test tubes and was sterilized at 121^{o}C for 15 mins. It was cooled and the LAB isolates were each inoculated into the sterile nutrient gelatin and were incubated at 25^{o}C for 7 days along with the control tube free from the test organism. After 7 days, the tube were placed in the refrigerator and later was brought out for gelatin liquefaction in which positive tubes turns liquid while negative tubes remains solid as control tube (Nabil *et al.,* 2004).

Selection of LAB for bio-preservation of nunu

The LAB isolates with the best anti-bacteria activity, good rate of acidification and high yield of antimicrobial compounds were selected and safety test such as heamolysis, gelatin liquefaction and DNAse were carried out on them and isolates that were negative for the three-safety test were selected as starter for bio-preservation of nunu.

Preparation and Pasteurization of milk

Fresh milk was aseptically collected from dairy farm at Bodija in Ibadan metropolis in a sterile McCartney bottles and was transported in an ice park to microbiology laboratory of University of Ibadan. The milk was pasteurized at 65^{o}C for 30 mins with constant homogenization [23].

Determination of the bio-preservative effect of lactic acid bacteria on nunu

The pasteurized nunu was cooled at room temperature. It was inoculated with *Lactobacillus* species singly and mixed at about, 6.0 x 10^{6} cfu/ ml. The un-inoculated nunu served as control. These, were stored at room temperature (30^{o}C). Afterward, the nunu was observed daily to determine when spoilage would start. The total microbial load was determined at the beginning of spoilage by serially diluting the samples and plating them out on Plate count agar. The plates were incubated at 35^{o}C for 48 h and the colony-forming units per millimetre of samples were determined. Sensory analysis of the nunu samples was carried out by nine-member panel of Judges consisting of students of the University of Ibadan who were familiar with the product. The organoleptic parameters used were colour appearance and odour. The rating were presented on 9-point Hedonic scale ranging from 9 = high acceptability to 1 = low acceptability where low acceptability indicates spoilage of the product [6].

Results and Discussion

The morphology, physiology and biochemical characterisation of lactic acid bacteria isolated from fermented cow milk is shown in table 1. The probable lactic acid bacteria isolates are;

Lactobacillus plantarum, *Lactobacillus pentosus*, *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus fermentum* and *Lactobacillus casei* all of which belong to the genus *Lactobacillus* thus, confirmed that, member of Lactobacillus species can be found in varieties of habitat including fermented foods and dairy products [6-8].

The zone of inhibition ranged from 6-12 mm in diameter. Two strains of Lactobacillus plantarum, Lactobacillus pentosus and Lactobacillus fermentum were able to inhibit the selected indicators at variable degrees, particularly; Lactobacillus pentosus and one of the two strains of Lactobacillus plantarum actively inhibited the growth of Staphyloccocus aureus (12 mm), Bacillus cereus (11 mm), Listeria monocytogenes (10 mm) and Escherichia coli (11 mm). The least zone of inhibition (6 mm) was produced by L. fermentum against P. aeruginosa. Similar findings was reported by Akabanda et al. [20] on antimicrobial activity of selected lactic acid bacteria isolated from nunu against Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi and Escherichia coli where variable degree of inhibition zones on the microorganisms were observed. According to Yang et al. [29], microorganisms, which produce antimicrobial compounds, are capable of inhibiting the growth of Gram-positive and Gram-negative bacteria. Such lactic acid bacteria antimicrobial effect were also reported by Adesokan et al. [7], where lactic acid bacteria cell free supernatant were used to inhibit the growth of Staphylococcus aureus, Pseudomonas, Candida albicans, Escherichia coli and Proteus vulgaris. Obadina et al. [27] also demonstrated the antagonistic effects of Lactobacillus plantarum against Staph. aureus, S. typhi, E. coli and B. subtilis.

The rate of acidification was taken at regular interval over a period of 24 hours incubation on selected lactic acid bacteria isolates and it was observed that the isolates showed variable rate of acidification (table 3). Lactobacillus pentosus and Lactobacillus plantarum B3 were the fastest acidifying LAB while the least acidifying LAB was L. plantarum after 18 h of fermentation. Lactic acid bacteria rapidly acidify fermented food products, which are advantageous towards preservation of food since growth of unwanted flora, could be inhibited in a low pH environment thereby enhancing safety and shelf life of food products [25]. Lactobacillus pentosus and Lactobacillus plantarum B3 were able to reduce the pH of fermented milk from 6.54 and 6.53 to 3.86, and 3.84 respectively after 12 h of fermentation revealing them as fast acidifier. The rate of acidification is very important in milk fermentation as it determines the aroma, texture and flavour of the product

[20]. Table 4 shows the proteolytic activity of selected LAB isolates. Lactobacillus pentosus and Lactobacillus plantarum B3 had the highest inhibition zone of 18 mm and 15 mm respectively, while the least proteolytic zone of 12 mm was observed for *Lactobacillus fermentum*. However, all the three strains of lactic acid bacteria had good proteolytic zone of clearance; an indication that they are capable of enzymatic breakdown of protein-lipid complex to protein which is further hydrolysed into smaller peptides. Thus, lactic acid bacteria with such property are presumed to have bio-functional activities such as antioxidants and anti-bacteria. The proteolytic activity of lactic acid bacteria in milk is important for the growth of these microorganisms as well as the development of organoleptic properties of the milk. According to Akabanda *et al.* [20], the production of a high quality fermented dairy products depends on the proteolysis of starter culture since, the products of proteolysis (peptidases and amino acids) contribute to the flavour enhancement of the milk product.

Table 1: Morphology, Physiology and Biochemical test for identification of Lactic Acid Bacteria Isolates

Test	SNN6	ONN1	SNN5	ONN7	BNN3	BNN4	ONN2
Gram reaction	+	+	+	+	+	+	+
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Ammonia from arginine	+	+	+	+	+	+	+
Gelatin hydrolysis	-	-	-	-	-	-	-
Casein hydrolysis	+	+	-	+	+	+	+
Spores formation	-	-	-	-	-	-	-
Growth at different NaCl concentration 2%	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+
4%	+	+	+	+	+	+	+
6.5%	+	+	+	+	+	+	+
Growth at Different Temperature 15^{o}C	+	+	+	+	-	-	+
30^{o}C	+	+	+	+	+	+	+
35^{o}C	+	+	+	+	+	+	+
45^{o}C	-	-	-	+	+	-	-
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Galactose	+	+	+	-	+	+	+
Sorbitol	+	+	+	+	±	±	+
Lactose	+	+	-	+	+	±	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	-	-	+	+	+
Mannose	+	+	+	+	+	+	+
Gluconate	+	+	+	+	+	+	-
Ribose	+	+	-	-	+	+	+
Salicin	+	-	-	+	-	-	-
Sucrose	+	+	+	+	+	+	+
Trehalose	+	+	-	-	+	-	-
Adonitol	-	+	-	-	-	-	-
Fucose	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-
D-raffinose	+	+	+	+	+	+	+
Probable organisms	*L. plantarum*	*L. pentosus*	*L.acidiphilus*	*L.fermentum*	*L. plantarum*	*L . paracasei*	*L.casei*

Table 2: Antagonistic activity of lactic acid bacteria against selected indicator organisms

LAB isolates	Indicator organisms/zone of inhibition in mm						
	Staphylococcus aureus	*Salmonella typhi*	*Escherichia coli*	*Bacillus cereus*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Listeria monocytogenes*
Lactobacillus pentosus	12.0	9.0	7.0	11.0	11.0	11.0	10.0
Lactobacillus plantarum	-	12.0	8.0	10.0	12.0	7.0	8.0
Lactobacillus fermentum	-	7.0	8.0	8.5	12.0	6.0	7.0
Lactobacillus plantarum B3	12.0	10.0	11.0	9.0	12.0	8.0	12.0

Table 3: Determination of the rate of acidification of LAB isolated from fermented cow milk at regular intervals

LAB isolates	Time (h)/pH				
	0	6	12	18	24
Lactobacillus pentosus	6.54	6.03	5.40	3.86	3.78
Lactobacillus fermentum	6.53	5.94	5.10	4.30	4.10
Lactobacillus plantarum	6.54	5.00	4.50	4.20	4.00
Lactobacillus plantarum B3	6.53	4.98	4.48	3.84	3.80

Table 4: Proteolytic activity of lactic acid bacteria isolated from fermented cow milk

LAB isolates	Diameter of proteolytic activity (mm)
Lactobacillus pentosus	18.0
Lactobacillus fermentum	12.0
Lactobacillus plantarum B3	15.0
Lactobacillus plantarum	16.0

The lactic acid bacteria isolates from fermented cow milk displayed variable degree of lipolysis as shown in table 5. *Lactobacillus pentosus* had the highest lipolytic activity while *Lactobacillus plantarum* B3 had the least. Though lactic acid bacteria are weakly lipolytic, but the ability of the

LAB strains to hydrolyse milk fat when used as starter culture may be of great advantage. Lactic acid bacteria are presumed to reduce cholesterol level when consumed in fermented milk. However, to ascertain such property, in-vivo study must be carried out on them [20]. Thus, this research work corroborates with the findings of Akabanda *et al*. (2014) who observed some of *Lactobacillus plantarum* isolated from *nunu* to have variable degree of lipolytic activity.

The single starter of *Lactobacillus pentosus* was observed to be effective in *nunu* preservation. This may probably be due to the capability of this LAB to produce organic acid thereby creating a low pH environment that could inhibit the growth of food spoilage bacteria. This is similar to the research carried out by Ying *et al.* [30] who reported that, *Lactobacillus pentosus* used as starter for bio-preservation of *pleurotus* species (edible mushroom), significantly inhibited the growth of spoilage microorganisms up to 17 days of fermentation. The shelf life of *nunu* was lower when the combined starter of *Lactobacillus plantarum* and *Lactobacillus pentosus* was used than the single starter of *Lactobacillus plantarum* B3 and *Lactobacillus pentosus*. This might be due to being strains of closely related species. According to Perin *et al*. [28], strains of the same species or closely related species are capable of producing antimicrobial compounds such as diacetyl, hydrogen peroxides, lactic acid and bacteriocin, which are capable of inhibiting the growth of spoilage microbes and that of the combined starter used in fermentation of foods.

Table 5: Lipolytic activity of lactic acid bacteria isolated from fermented cow milk

LAB isolates	Diameter of lipolytic activity (mm)
Lactobacillus pentosus	7.0
Lactobacillus fermentum	-
Lactobacillus plantarum B3	3.0
Lactobacillus plantarum	5.0

Table 6: Shelf life monitoring of nunu (log_{10} cfu/ml)

Isolates	Day 3	Day 4	Day 5	Day 6	Day 7
L. plantarum	nd	1.57 ± 0.14^{a}	1.65 ± 0.35^{a}	1.95 ± 0.28^{a}	2.13 ± 0.03^{a}
L. plantarum B3	nd	1.59 ± 0.20^{b}	1.58 ± 0.21^{b}	1.60 ± 0.01^{b}	1.65 ± 0.02^{b}
L. pentosus	nd	nd	nd	1.59 ± 0.01^{b}	1.60 ± 0.02^{b}
L. plantarum +*L. pentosus*	nd	1.55 ± 0.03^{a}	1.64 ± 0.35^{a}	1.85 ± 0.14^{a}	2.01 ± 0.00^{a}
L. plantarum B3+ *L. pentosus*	nd	nd	1.55 ± 0.10^{b}	1.57 ± 0.03^{b}	1.72 ± 0.03^{c}
Un-inoculated	1.65 ± 0.18^{a}	1.79 ± 0.01^{a}	2.01 ± 0.02^{a}	2.30 ± 0.28^{a}	2.25 ± 0.35^{a}

Mean value within the same column with the same superscripts are not significantly different ($p<0.05$

Conclusion and Recommendation

Lactic acid bacteria which are known to be generally regarded as safe has been used various fermentation processes. Their ability to survive an-aerobically and release metabolites in fermenting medium has been of significant importance in food fermentation. *Nunu* is spontaneously fermented cow milk whose shelf life is low and nutrients are lost within 24 hours of fermentation. Thus, this research is vital because, functional properties of the isolated and characterized lactic acid bacteria can be used to improve preservatives effect, flavour additives and taste of fermented milk products particularly nunu. The isolated strains may positively have impact on their use as starter culture for traditional fermented *nunu*, with a view to improving shelf life, nutritional qualities and safety of nunu so produced. Hence, these organisms could be recommended for use as starter for bio-preservation of *nunu* if further work is done to establish its potential use as bio-preservative for *nunu* production. Since *nunu* has been reported to have nutritional advantage as yogurt, large-scale production could be encouraged.

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