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Developing Starter Culture from Lactic Acid Bacteria Isolated from Cow Milk for the Production of Nigerian "Nunu"

Adedayo, M. R.; and Abdulkareem, T. O.

Department of Microbiology, Faculty of Pure and Applied Sciences, Kwara State University, Malete, P.M.B. 1530, Nigeria.

Corresponding author: orlanre1988@gmail.com

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Abstract

Starter cultures are mostly organisms deliberately introduced into food as single culture or mixture of cultures to bring about fermentation and production of desired characteristics in food through metabolic and enzymatic activities. Organisms in this category have attained the generally regarded as safe (GRAS) status, are majorly lactic acid bacteria with food acidification and production of natural preservatives potential. This research was aimed at developing starter culture for the production of local dairy drink "Nunu" Cow milk was obtained from "Gaa Mobolohunduro", Tanke, Ilorin, Kwara State, Nigeria. Potential lactose fermenters were isolated on MRS agar in pour plate technique. The isolates were characterized and identified using standard procedures. Lactic Acid Bacteria isolated were inoculated as starter culture for milk fermentation and the products were subjected to pH, titratable acid and organoleptic assessments following standard procedures. Two of the isolated lactic acid bacteria with best fermentation products were identified through molecular technique. A total of six isolates were obtained and identified as *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Lactobacillus* spp. The pH ranges from 5.46±0.01 to 6.03±0.01while the titratable acidity ranged from 0.244±0.004 to 0.526±0.006. The moisture content ranged from 90.50±0.30 to 91.75±0.35. The fermentation product from the isolated *Lactobacillus* sp. scored 80 % during the organoleptic assessment. Molecular evidence confirmed the two isolates as Lactic Acid Bacteria. In conclusion, isolated species could be used as potential organisms for milk fermentation.

Keywords: Cow Milk, Nunu, Lactic Acid Bacteria, Starter Culture, *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus*.

Introduction

Lactic acid bacteria (LAB) are an industrially important group of bacteria and used as starter cultures for the production of fermented milk products (e.g. yoghurt and some cheeses) in the dairy industry. LAB are gram positive, non-sporeforming, catalase negative bacteria, and contain the following genera: *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus* and *Weissella*. These groups of bacteria have been isolated from the raw materials of food, such as raw milk, plants, vegetables and fruits (Karakas-Sen and Karakas, 2018).

Nono (Milk) is an exceptionally nutritive dairy product that provides an ideal niche for lactic acid bacteria (LAB) such as *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Enterococcus* to thrive in. The milk microbiota of an animal host delineates health status of the organism. Although recognition on the benefit of probiotic has grown in the last few decades, which led to the rapid expansion on the world of probiotic, the Lactobacillus genera is still one of the most widely known and applied probiotic (Andrian *et al.,* 2018).

Bacteriocins, ribosomally synthesized antimicrobial peptides produced by various bacteria, display antimicrobial activity against closely related bacterial strains, and are widely used as food preservative in food industry, in agriculture and veterinary medicine as a therapeutic. These peptides are sensitive to specific proteolytic enzymes and can be heat stable. Bacteriocin production ability was widespread among LAB isolated from raw milk. The strains producing bacteriocin nisin were most abundant among LAB isolated from raw cow, ewes and goat milks. Furthermore, lactococci producing lacticin 481 and enterococci producing enterocin AS-48 have also been found at high incidence. *Lactococcus lactis* strain producing bacteriocin nisin Z was isolated from a traditional fermented Nigerian dairy product, wara that made from raw cow milk (Karakas-Sen and Karakas, 2018).

To develop a relatively stable product of desired organoleptic property, with enhance nutrient and microbiologically safe, there is the need to develop and use starter culture. Previous reports on nunu have been concentrated on organisms involve in its production and or spoilage. This research was hence, conducted to develop a starter culture for the production of fermented dairy milk in Nigeria.

Statement of the Problem

Global food safety agencies envisaged a world of healthy foods with natural food preservatives, which are generally regarded as safe (GRAS). Sarter culture status as GRAS, has led to increase detailed studies on the potency and application of other starter culture as natural alternative substance for food preservations.

Study Objectives

- Isolate bacteriocin producing bacteria from fresh cow milk;
- Determine biochemical and molecular characteristics of the isolates;
- Assess the isolates as starter culture for fermented dairy milk;

Materials and Methods

Collection of Fresh and Powdered Milk Samples

Milk sample was collected from cow through the udder in the early morning hours in sterile containers and transported in ice pack to the Microbiology Laboratory of Kwara State University, Malete for analysis. Also, five hundred gram (500 g) of powdered milk was purchased from a provision store in Ilorin Kwara State for the research.

Isolation of Lactic Acid Bacteria using Pour Plate Method

Lactic acid bacteria were isolated from the cow milk using de Mann Roggossa (MRS) agar. The colonies obtained were counted using colony counter model (LT-37) (James et al., 2017). Subculturing was carried out repeatedly to obtain pure culture. Six isolates were obtained in pure culture. The pure cultures were maintained at 4 °C in a refrigerator for further use (Bali et al., 2011).

Characterization and Identification of Isolates

Isolated bacteria were characterized and identify using colonial morphology, Gram's reaction and biochemical tests (Cheesbrough, 2005). Molecular characterization and identification of two of the isolated bacteria (LAB 5 and 6) was done using PCR technique, gene sequencing and comparing the gene with a specific primer of LAB. The DNA of the isolates were extracted and sequenced. Identification of the isolates was through sequencing of the amplified 16S rRNA gene and its homology search using BLAST. The agarose gel electrophoresis of 16SrRNA amplified genes of isolates was run with DNA from *Lactobacillus* species.

Phenol Tolerance Test on Isolates

MRS broth containing 0.1, 0.2, 0.3 and 0.4 % of phenol concentration were prepared for the determination of phenol tolerance by isolates. Fresh culture of isolates was inoculated and incubated at 37 °C for 48 hours. Only the medium was used as negative control. Results were determined by observing turbidity after 24 and 48 hours. Positive result was indicated by turbidity and no growth was found in negative control (Sultana, 2017).

Test for Diacetyl Test Production by Isolates

Five mililitre (5 ml) of peptone water, containing 1 ml alpha naphthol and 1 ml KOH was dispensed into clean sterile test tubes. The tubes were inoculated with the test organisms and incubated at 37 °C for 48 hours. A strong red ring formed indicates positive reaction (Sultana, 2017).

Preparation of McFarland Turbidity Standards

Precisely 0.5 % McFarland turbidity standards was prepared by mixing 99.95 sulfuric acid and 0.5 barium chloride to obtain solutions with specific optical densities. Zero-point five McFarland turbidity standard provides an optical density comparable to the density of a bacterial suspension of about 1.5x 10^8 colony forming units (CFU/ml) (Microbe Online, 2021).

Fermentation Procedure using the Isolated Lactic Acid Bacteria

Overnight culture of the LAB isolates in peptone water was adjusted to the turbidity of 0.5 % Macfarland containing approximately 10^8 Cfu/ml. The adjusted culture was used as inoculum for fermentation. The isolates were centrifuged at 4000 rpm for 10 mins and

washed twice with sterile distilled water to remove the media from the cell (Sood *et al.*, 2011).

Re-Inoculation of LABs Isolates into Fresh Milk for Fermented Milk Production

Fermentation was carried out as described by (Akabanda *et al.*, 2014) with slight modification using the isolated organisms as inoculum. Five sterile container labeled A, B, C, D and E were used as fermentation jars. The isolates were used in combination of two at concentration ratio of 1:1 v/v of each in five fermentation jars. One hundred grams (100 g) of powdered milk was weighed into each container. The powdered milk was dissolved by adding 200 ml of sterile distilled water at room temperature. The mixture was thoroughly mixed by stirring gently with a sterile stirrer to aid dissolution. Eight hundred milliliters (800 ml) of distilled water at 100 °C was poured into each mixture and pasteurization was done at 70 °C for 1 minute. The pasteurized mixture was cooled to about 45 °C in a water bath. Ten milliliters (10 ml) each of inoculum (containing 5 ml of each bacterium) was used under sterile condition. Fermentation jar D and E were negative (All the isolates were used) and positive (No inoculation) controls. The containers were sealed to avoid contamination. The mixtures were left to ferment for 12 hours. After fermentation, the products were subjected to further analysis.

Physicochemical Analysis of the Fermented Milk Samples

The physicochemical parameters considered were titratable acidity, moisture content and pH.

Titratable Acidity

Ten millimeters each of the fermented milk produced was added to 90 ml distilled water. Twenty-five (25 ml) of it was measured in a conical flask. Two drops of phenolphthalein were added. Fifty (50 ml) of NaOH was measured in a burette fixed to a retort stand. The amount of acidity in the samples was determined by titrimetric method as described by (Egbere *et al.*, 2008). The total titratable acidity was determined by the amount of 0.1 M sodium hydroxide titrated against 25 ml of the sample. The titratable acidity was calculated using the formula: Titratable Acidity=Titre value x 0.09 (Adesokan *et al.*, 2013).

Moisture Content

Five millimeters each of the fermented milk produced was added into a crucible and was kept in the oven at 100 °C for 1 hour. the moisture content was calculated thus;

Moisture content= $(W_1-W_2)/W_1$

Where W_1- weight of crucible + sample

W_2- weight of crucible + sample after 1 hour.

pH

The pH of the sample was determined using a pH meter (Denver model 20). The pH meter was turned on. The electrode was cleaned and calibrated. The electrode was placed in 25 ml of the sample. The reading was recorded and the electrode was rinsed with distilled water. The same procedure was repeated for all samples.

Organoleptic Characteristics of the Fermented Milk

A twenty men semi trained panel was constituted to carry out the organoleptic and sensory assessments of the products based on appearance, smell, consistency and taste on a hedonic scale of 1-5. The percentage score was calculated based on the record obtained from the panel members.

Statistical Analysis

The data was shown as the mean ± standard deviation (SD, n = 5). The results obtained was analyzed using SPSS 18.0 program for Windows (Munich, Germany) and by analysis of variance (ANOVA) with significance level set at p = 0.05.

Results

Morphological Characteristics of Isolates

In the current study, LAB was isolated from raw milk sample. the isolated LAB species were Gram-positive, rods or cocci (Table 1).

Biochemical Characteristics of Isolates

The biochemical characteristics of the isolates were as presented in Table 1. The isolates were identified as *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Lactobacillus* sp.

Molecular Characteristics of the LAB Isolates

On the basis of sequencing of the amplified 16S rRNA gene and its homology search using BLAST; the LAB isolates were identified as *Lactobacillus* sp. The agarose gel electrophoresis of 16SrRNA amplified genes from *Lactobacillus* sp. was presented in Figure 1.

Physicochemical Parameters of the Fermented Milk Produced by LAB Isolates

Physicochemical parameters of the fermented milk produced by LAB isolates is presented in Table 2. The fermented milk products had pH ranges between 5.45 to 6.04, moisture content between 90.2 to 91.4 % and titratable acidity of 0.24 and 0.532.

Table 1: Biochemical Characteristics of the LAB Isolates from Fresh Cow Milk Sample

Isolates	Gram' s Reactions	Shape	Catalase	Oxidase	Urease	Methyl red	Voges proskauer	Coagulase	Citrate	Indole	Glucose	Lactose	Sucrose	Isolated Organisms
LAB1	+	Cocci	-	+	+	+	-	-	+	-	+	+	-	*Lactococcus* sp.
LAB2	+	Cocci	-	_	+	+	-	-	+	-	+	-	+	*.Leuconostoc* sp.
LAB3	+	Cocci	-	-	-	+	-	-	-	-	+	-	+	*Leuconostoc* sp.
LAB4	+	Cocci	-	-	-	+	-	-	-	-	+	+	+	*Pediococcus* sp.
LAB5	+	Rod	-	-	+	+	-	-	+	-	+	+	+	*Lactobacillus* sp.
LAB6	+	Rod	-	-	+	-	-	+	+	-	+	+	+	*Lactobacillus* sp.

Key: LAB = Lactic acid bacteria; - Negative; + Positive

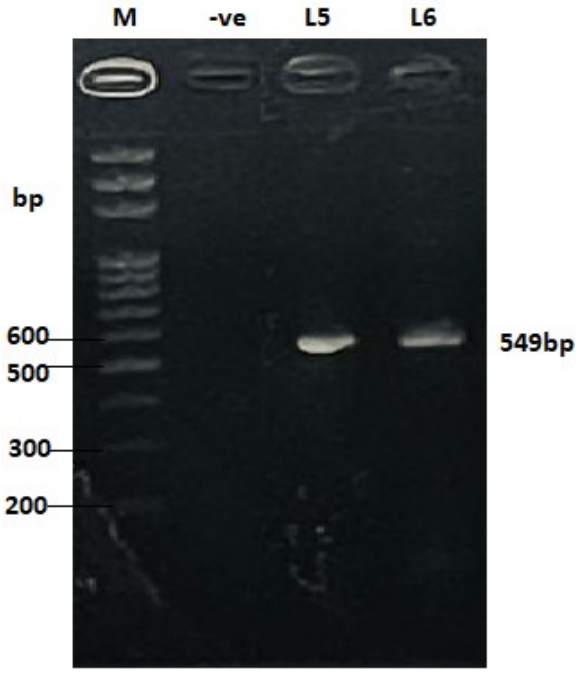

Figure 1: Agarose gel electrophoresis of 16SrRNA amplified genes from *Lactobacillus* sp.

The isolates are L5 and L6 as indicated on the gel with 549 bp.

Table 2: Physicochemical Properties of the Fermented Milk Products

FermentedMilk Samples	pH	Titratable Acidity %	Moisture Content %
A	5.46 ± 0.01[a]	0.526 ± 0.006[a]	91.50 ± 0.30[a]
B	6.03 ± 0.01[b]	0.244 ± 0.004[b]	90.80 ± 0.10[b]
C	5.63 ± 0.01[c]	0.476 ± 0.004[c]	91.75 ± 0.35[c]
D	5.66 ± 0.01[d]	0.440 ± 0.004[d]	91.70 ± 0.70[d]
E	5.92 ± 0.01[e]	0.276 ± 0.004[e]	90.50 ± 0.30[e]

Key:

A= milk fermented with LAB 1+2

B= milk fermented with LAB 3+4

C= milk fermented with LAB 5+6

D= milk fermented with all LAB isolates

E= Uninoculated milk

Note: Data are means of two replicates ±SD

Table 3: Organoleptic Attributes of the Fermented Milk Products

Fermented Milk Samples	Appearance	Smell	Consistency	Taste	Total	General Acceptability %
A	34	33	30	31	128	64
B	32	31	29	30	122	61
C	40	44	35	41	160	80
D	33	31	40	32	136	68
E	30	0	0	0	30	15

Key:

A= milk fermented with LAB 1+2

B= milk fermented with LAB 3+4

C= milk fermented with LAB 5+6

D= milk fermented with all LAB isolates

E= Uninoculated milk

Discussion

The present investigation highlighted the isolation and characterization of LABs and the use as starter culture in the production of fermented milk, nunu. Four genera of lactic acid bacteria (LAB) (*Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Lactobacillus* sp.) were obtained during this study. The presence of these genera of lactic acid bacteria in dairy and fermented products generally have been previously documented (Jirayu *et al.*, 2021). Their roles in lactic acid fermentation as homolactics is highly desirable and variously explored in the food industries for food processing and preservation. Ability of these organisms to

produce antimicrobials against food pathogens and flavour enhancing compounds among others have also been reported (Akpi *et al.*, 2020).

The genera isolated are among the organisms that have attained the Generally Regarded as Safe status and hence well accepted in food processing (Makarova *et al.*, 2006; Zheng *et al.*, 2020). They are found as normal mutual with human and they occur in dairy products as well as unprocessed milk (Martin *et al.*, 2013; Duar *et al.*, 2017). Some members of this group of organisms are utilized in production and as probiotic in food such as yoghurt, their presence enhances nutritional and health benefits ((Sutana *et al.*, 2016; Inglin and Raffael, 2017). *Lactobacillus* species are known to play key roles in producing desirable taste, aroma, consistency and good physical appearance in fermented milk products (Salvetti *et al.*, 2012; Vyas *et al.*, 2014; Rao *et al.*, 2015; Akpi *et al.*, 2020). Fermentation using starter culture gives product with distinct and unique organoleptic and physicochemichal characteristics. However, spontaneous fermentation yields product with varied property (Fagbemigun *et al.*, 2021).

The use of these isolates as starter culture in milk produce fermented products of desirable qualities. The physicochemical and organoleptic characteristics were well accepted and highly scored to meet consumer's taste. The identity of the two *Lactobacillus* species that yielded the best fermentation products (Product C; Table 5) was further confirmed using molecular techniques for the characterization. The two isolates were confirmed as *Lactobacillus.*

The pH of fresh unfermented milk is near neutral. The titratable acid (TA) of the products increased as a result of the acidic fermentation that has taken place in the milk with the introduction of the isolated *Lactobacillus* as starter culture. Increase in acidity during fermentation has also been reported by Achi and Akobor (2006; Fagbemigun *et al.*,2021). The acid content of fermented foods enhances digestibility and microbiological safety (Livia, 2004). It also favours the growth and metabolism of *Lactobacillus* species. Acid and diacetyl production by the isolated *Lactobacillus* species have desirable qualities impacted on the products. This will foster long shelf life and elimination of food borne microbial pathogens.

The organoleptic assessment revealed that the product shared characteristics with earlier report on similar study. Sensory attributes of the fermented milk produced were similar to earlier reports on fermented milk (Akabanda, 2009).

Conclusion

In conclusion, the lactic acid bacteria isolated could be potential organisms for use as starter culture in milk fermentation and the bacteriocin produced is potent against the test organisms such as *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis*.

Recommendation

More work should be done on the suitability of *Lactobacillus* sp. for use as starter culture.

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