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Full Length Research Article

ISOLATION, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF LACTIC ACID BACTERIA FROM WHEY OF A NIGERIAN TRADITIONALLY FERMENTED YOGHURT DRINK

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ABSTRACT
Lactic Acid Bacteria (LAB) have been identified as an essential group of microorganisms due to the health-promoting effects they exert on human and animal hosts. This research was conducted to, isolate LAB from whey obtained from *kindirmo* a locally fermented cow milk product in Nigeria, characterize the strains based on physiological and biochemical properties and identify them using 16SrRNA sequencing. A total of 32 samples were collected aseptically and the whey cultured on an MRS and M17 media. Physiological and biochemical results showed that the isolated organisms, which were mostly rod and cocci shaped, included gram positive and catalase negative species. The organisms did not only vary in their abilities to tolerate and grow at different concentrations of pH, temperature and NaCl, but were able to ferment twelve different sugars distinctively. The ten most desirable strains obtained were subsequently screened by molecular techniques using PCR and sequence analysis. The PCR results revealed that 98% of the identified organisms were *Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus acidophilus, Streptococcus thermophilus, Lactobacillus gasseri* and *Lactobacillus plantarum*. These findings showed that *kindirmo* could be an excellent and potential source of probiotic bacteria, which are often the main sources of probiotics. Further screening and identification processes were recommended to ascertain the functional, technological and probiotic properties of the strains.

Keywords: *kindirmo*, lactic acid bacteria, fermentation, whey, probiotics.

INTRODUCTION

Different types of fermented foods have been traditionally produced and consumed for many centuries across diverse cultures depending on the peculiarity of the geographical location (Heinen *et al*., 2020). As a result of the overwhelming functional and technological properties exhibited by Lactic Acid Bacteria (LAB), they have, over time, been a subject of interest to most researchers in the dairy, pharmaceutical and agricultural industries. LAB have been isolated from numerous fermented food products of both plant and animal origin for their nutritional and technological benefits as well as for use as probiotics and functional food resources (Grajek *et al.,* 2005; Chalat *et al*., 2011). They are special and unique groups of gram positive, catalase negative, facultative anaerobic, non-spore forming organisms that produce only lactic acid bacteria as the end product of fermentation (Bassyouni *et al*., 2012).
Essentially, milk is recognized as one of the intrinsic environments for LAB proliferation (Widyastuti and Febrisiantosa, 2014). Milk from diverse mammalian animals has been utilized in dairy fermentation to generate numerous products of interest usually by the actions of lactic acid bacteria widely employed for most milk fermentation (Oyedokun *et al*., 2022). Microbial activity spoils milk if left untreated within a short period of time and as a result, processing it advances its storage and broadens its utilization (Egwim *et al*., 2015).Some examples of the local drinks relished in Africa, particularly in Nigeria, which contain LAB, include *ogi* (a fermented cereal porridge from West Africa, typically made from maize, sorghum, or millet); *kunun zaki* (an indigenous Nigerian drink made from whole grains of millet, sorghum or corn); *nono* (a spontaneously fermented yoghurt-like product in Nigeria and other parts of West Africa); *kindirmo* (a Nigerian indigenous yoghurt drink), and *wara* (an unripen cheese prepared by coagulating cow milk with the leaf extract of *calotropis procera* also known as sodom apple in addition to the well-known yoghurt drink (Agarry *et al.*, 2010; Franz *et al*., 2014; Igwegbe *et al*., 2015; Kassum *et al.,* 2019; Oyedokun *et al*., 2023). Most of the prominent LAB found in some of these local milk products includes *Bifidobacterium longum, Lactobacillus gasseri, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus pentosus, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus casei, Lactobacillus gastricus,* and *Lactobacillus salivarius* (Holzapfel, 1997; Shah, 2000; Plessas *et al*., 2012; Jamyuang *et al*. 2019; Łubiech and Twarużek 2020). The Fulani herdsmen in Northern Nigeria have been known for the production of *kindirmo* (Bamgbose *et.al*., 2022). The process of which includes collection of raw milk from lactating cows, boiling and cooling of the milk followed by overnight fermentation. Due to the fact that the fermentation process of *kindirmo* is usually spontaneous, the product normally contains diverse community of microorganisms performing different functions during the fermentation process. The *kindirmo* may also have the probiotic benefits outlined by Plessas *et al.* (2012).

As LAB are widespread in nature with many species finding application in the food and pharmaceutical company, the conventional biochemical and physiological tests frequently used in identifying them clearly have some limitations in discriminating large number of isolates showing similar physiological characteristics (Adejumo, 2014). Many studies have thus focused on the application of molecular techniques for the identification of Lactobaccilli (Chagnaud *et*. al. 2001). Sequencing analysis of the 16SrRNA gene was therefore used in this study to determine the diversity of LAB found in these traditional foods in order to produce excellent quality fermented products that will not only offer the desired beneficial effects to consumers (Perdigon *et al*., 2001), but also meet the international standard requirements for such

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products.
Whey, which is the liquid component that separates from milk after cuddling during yoghurt and cheese processing, is a by-product and widely perceived to be a waste. It however contains valuable proteins such as alpha lactalbumin and beta lactoglobulin, with many nutritional, chemical and biological properties (Trindade *et al*, 2019). This study was therefore designed to isolate LAB from the whey obtained from *kindirmo* in order to harness its worth.

STUDY AREA AND METHODOLOGY

Description of the Study Area

This study was conducted in Abuja, the Federal Capital Territory (FCT) of Nigeria, situated in the center of the country and currently made up of six area councils, namely: Abaji, Amac Bwari, Gwagwalada, Kuje and Kwali (Abubakar, 2014). Samples of kindirmo were randomly purchased from Fulani vendors (as they are the only producers and suppliers of the product to the consumers), from various locations in the FCT namely: Kuje, Gosa, Lokogoma, Giri, Lugbe, Kwali and Garki (**Figure 1**). The samples were always collected during the early hours of the morning from the selected locations; and a total of thirty two (32) samples were collected in sterile plastic bottles, labeled and transported in previously sterilized insulated plastic coolers containing ice blocks, to the Fermentation and Bioprocessing Laboratory at the National Biotechnology Development Agency, Abuja, for all the analysis.

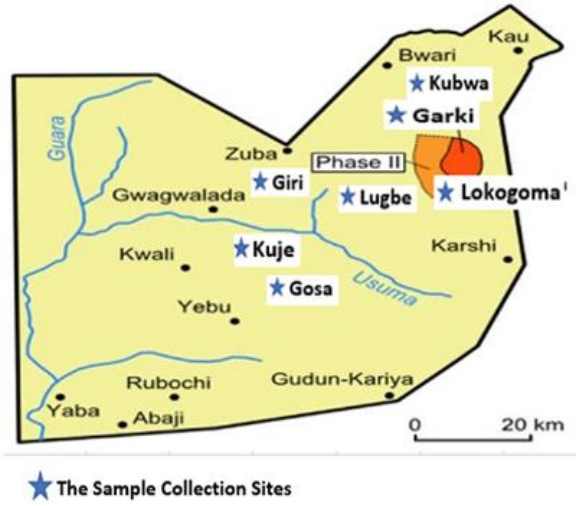

Fig. 1: Physical Map of the Federal Capital Territory (FCT) Abuja, indicating the Sample Collection Sites (Source: Abuja Geographic Information System (AGIS)

Whey Preparation

The whey was prepared by aseptically pouring the *kindirmo* into a folded Whatmann™ Grade 1 filter paper (diameter 150mm), suspended in a small sterilized beaker covered neatly with a clean foil paper and stored in the refrigerator for 24 hours. The drained liquid obtained (the whey) in the beaker was kept on the shelf at 37°C for 1 hour before being used for the isolation.

Isolation of LAB

The bacterial isolation was carried out by serially diluting the whey samples with sterilized peptone water. One milliliter (1mL) of the whey sample was pour-plated on MRS (containing per liter; beef extract, 2 g; tryptone, 10g; yeast extract, 4 g; glucose, 10 g; $K_2HP0_4.3H_20$, 2.5 g; sodium acetate, 5 g; tris ammonium citrate, 2 g; $MgS0_4.7H_20$, 200 mg; manganese sulphate, 50mg; Tween 80, 1 ml) and M17 (containing phytone peptone, 5.0g, polypeptone, 5.0 g; yeast extract, 2.5 g; beef extract, 5.0 g; lactose, 5.0 g; ascorbic acid, 0.5 g; GP, 19.0 g; 1.0 M $MgSO_4 \cdot 7H_2O$, 1.0 mL) media ;which were previously sterilized by autoclaving at 121°C for 15minutes and allowed to cool to 40 ^{0}C before being used for the selective isolation of Lactobacilli and Streptococci bacteria respectively. The plates were incubated anaerobically at 37°C for 48 hours using a gas jar pack (Misganaw and Teketay, 2016).

Phenotypic Identification of the Isolates

After the incubation period, discrete colonies obtained were phenotypically identified based on conventional morphological and biochemical characteristics. Gram staining (determined by light microscopy) followed by catalase test (conducted by adding 3 % hydrogen peroxide on the colonies on a clean glass slide) and motility test (in sulphide indole motility medium) were carried out according to the method of Cheesbrough, (2006). Gram positive and catalase negative isolates were subsequently purified by sub-culturing, using the streak plate technique

Determination of the Microbial Growth at Different Temperatures

Five milliliters (5mL) of the MRS broth containing bromocresol purple as indicator was prepared, transferred into glass tubes and sterilized by autoclaving at 121°C for 15 minutes and then allowed to cool. Fifty micro liters (50µl) of overnight grown cultures were introduced into the MRS broth and incubated for 3 days at varying temperatures of 15°C, 37°C, and 42°C. During the incubation time, cell growth at any of the temperatures was observed by the change in color of the basal medium from purple to yellow (Hawaz, 2014).

Determination of Growth at Different NaCl Concentrations

Isolates were tested for their tolerance to different levels of NaCl concentrations, namely: 2%, 4% and 6.5%. In this process, five (5 mL) of MRS broth containing bromocresol purple indicator were prepared using the various concentrations of the NaCl and transferred into different tubes. These tubes were inoculated with 50 µl of previously overnight grown cultures and then incubated at 37°C for 3 days. The change in color of the basal medium from purple to yellow was considered as proof of cell growth (Hawaz, 2014).

Gas Production from Glucose

The homofermentative and heterofermentative capabilities the isolates were determined using the CO_2 production from glucose. In this process, an MRS broth was prepared, inoculated with 50µl of freshly prepared cultures, with addition of two drops of bromocresol purple indicator and inverted Durham tubes. The test tubes were then incubated at 37°C for 3 days. Gas production as depicted by a displacement of the Durham tubes during the 3 days was evidence of utilization of the glucose and CO_2 production from it, indicating a hetero-fermentative pathway while non-displacement of the tubes with only a color change of the basal medium from purple to yellow was an indication of acid production and suggestion of the presence of a group of homo-fermenters (Bassyouni *et al*., 2012).

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Carbohydrate Fermentative Spectra

The resulting isolates from the above tests were purified by streaking on sterilized and cooled MRS agar and tested for sugar fermentation profile using eleven sugars (arabinose, dextrose, jlactose, maltose, mannitol, mannose, rhamnose, sucrose, sorbitol, cellobiose and xylose).

The strains that produced a change in color of the basal medium from purple to yellow, an indication of sugar utilization, were selected and stored on agar slants at 4^{o}C until needed for the molecular confirmation protocols.

DNA Extraction and Molecular Identification of isolates

Genomic DNA Extraction

The genomic DNA extraction of the selected LAB was done as described by Ayodeji *et al*. (2017). In this process, the selected LAB isolates were grown overnight in MRS and M17 broths for Lactobacilli and Streptococci, respectively. The cells in the broth were pelleted by centrifugation at 4000 rpm for 10 min. The pellets were then washed twice with TE buffer (10-mM Tris-Cl, 1-mM EDTA, pH 8.0). The genomic DNA of the isolated strains was extracted using the guanidium thiocyanate–N-lauroylsarcosine denaturing method. The quantity and the purity of the total DNA were verified using agarose gel electrophoresis, and the DNA was stored at − 20°C until needed (Drancourt *et al.* 2000).

16SrRNA Amplification and Sequence Data

The method of Drancourt *et al*. (2000) was also employed for 16S rRNA amplification. The PCR assays were performed in an automated temperature cycling device (Test Kit, China), using 5 µl of extracted DNA, 25-µl NzyTaq 2× Green Master Mix (Genaxxon Bioscience, Germany) and 2 µl of each primer in a total volume of 50µl. The amplification cycling program consisted of a 5-min initial denaturation at 94°C, followed by 35 cycles of a 2-min denaturation at 94°C, a 1-min annealing at 51°C, and a 2-min extension at 72°C, with a final extension at 72°C for 5 min as reported by Plessa et al. (2017). The amplified fragments were verified by electrophoresis on 1% (w/v) agarose gels stained with 0.5 µg/mL ethidium bromide. Fragment sizeswere verified as positivefor the universal 16S rRNA gene. These were then sent to Incaba Biotech West Africa, Nigeria for sequencing using an automatic sequencer. The raw sequences were manually base-called using the BioEdit software and nBLAST searches were performed using the GenBank Internet server (http://www.ncbi.nlm.nih.gov) for comparison with other strains deposited in the public databases to identify the species of each isolate. Sequences that showed more than 98% similarity were considered as belonging to the same taxonomy unit.

RESULTS AND DISCUSSION

Lactic Acid Bacterial Identification

Dairy products have long been referred to as functional foods due to the fact that they are good sources of nutrients that have health promoting activities, such as vitamins and minerals. Notwithstanding, research is now focusing specifically on other components in dairy products. Fermented milks, though considered to be good sources of vitamins and minerals, also supply lactic acid bacteria (LAB) that could exert additional health benefits on the consumers (Plessas *et al.,* 2012). To be able to play this vital role, adequate numbers of viable cells of LAB of more than 10^6 CFU/mL need to be consumed regularly to exert probiotic benefits to the consumers (Mortazavian *et al*. 2012). In this study, the LAB isolates were presumptively identified as Lactobacilli and Streptococci using their morphological and biochemical characteristics based on their colors (white to milky and yellowish), sizes (small to large), shapes (rod and cocci), and appearances (circular margin) on the MRS and M17 media. Also, the gram reaction of the strains revealed that all the bacteria were either gram-positive rod or cocci having different arrangements from chain, paired and tetrads, marked by purple color; the same way the catalase reaction were negative for all the isolates as observed by the absence of oxygen bubbles (Table 1). This result is in agreement with the research carried out by Bamgbose *et al*. (2022). The results of the 32 samples collected revealed that out of a total of 132 strains, 24 lactic acid bacterial strains were obtained, of which 19 isolates representing 79% were *Lactobacillus* spp. while 5 isolates representing 21% were *Streptococcus* spp.

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Table 1: Results of Presumptive Morphological and Biochemical Characterization of the LAB Isolates

Tests	**Selected LAB Isolates**									
	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
Gram Stain	+	+	+	+	+	+	+	+	+	+
Cell Morphology	R	C	C	R	R	R	R	R	R	R
Catalase	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-
Gas from Glucose	-	-	-	-	-	-	-	-	-	-
NaCl Concentration:										
2 %	+	+	+	+	-	-	-	-	+	+
4 %	+	+	-	-	+	+	-	-	+	+
6.5 %	+	+	-	-	+	-	-	+	+	+
Growth at different Temperatures:										
15 ^{0}C	-	-	-	-	-	-	-	-	+	+
37 ^{0}C	+	+	+	+	-	+	+	-	+	+
42 ^{0}C	+	+	+	+	+	+	+	+	+	+
Carbohydrate Utilization:										
Arabinose	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	-	-	-	+	+	+	-
Mannitol	-	-	-	-	+	+	+	-	-	-
Mannose	-	+	+	+	+	-	-	-	+	+
Rhamnose	+	+	+	-	-	-	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	-	-	-	+	-	-
Cellobiose	-	-	-	-	-	+	-	-	-	-
Xylose	-	-	-	-	-	-	-	-	-	-
Presumptive Isolate	Lb	St	St	La	Lc	Lg	Lp	Lb	La	Lc

Lb=*Lactobacillus bulgaricus*; St=*Streptococcus thermophillus*; La = *Lactobacillus acidophillus*; Lg=*Lactobacillus gasseri*; Lc = *Lactobacillus casei*; Lp= *Lactobacillus plantarum*
+ = positive; - = negative; R = rod; c = cocci.

Molecular Identification of the Isolates

Molecular identification of the selected isolates was carried out by amplification and sequencing of their 16SrRNA gene. Amplified selected LAB DNA showed distinct single DNA bands with molecular weight of 1000 bps (**Fig. 4**). The amplified PCR products were purified, sequenced, and aligned using a blast with the published sequences of the 16SrRNA genes of other strains deposited in NCBI databases. According to the blast results of the isolates with more than 99% similarities, they were identified as *Lactobacillus plantarum, Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei* and *Lactobacillus gasseri;* with the confidence degree of E = 0.0, homology of between 99 and 100% for all the isolates. Their partial sequences were submitted to the GenBank (https://www.ncbi.nlm. nih.gov/genbank). at NCBI database with accession numbers KJ: MH656840.1; GS: MT545060.1; LK: HM218349.1; GR: HG423864.1; LG: MN796080.1; GK: KP763901.1; KL: MW463614.1 (**Table 2**).

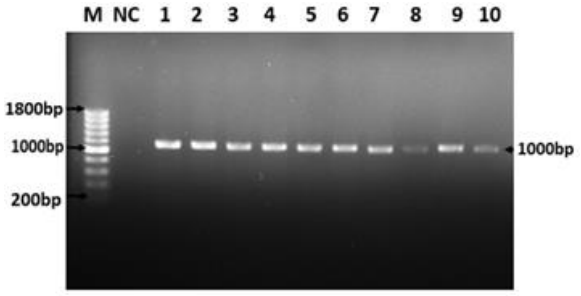

Figure 4: Agarose gel 1% (w/v) showing the PCR products amplified from 16S rRNA gene
(1000bp) of the selected LAB isolated from whey samples. M = DNA molecular marker; NC = Negative Control; Lane 1-10 = Presumptive Isolates (KJ03, GS26A, LK23, GR41, LG04, GK21, KL06, GR29, NS16 and MN41).

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Identified Isolates	Location code*	Frequency of Occurrence	Accession No	% Identification
Lactobacillus plantarum	KJ03	1	MH656840.1	99.79
Streptococcus thermophilus I	GS26A	1	MT545060.1	99.95
Streptococcus thermophilus II	LK23	1	HG423864.1	93.55
Lactobacillus bulgaricus	GR41	2	HG423864.1	93.48
Lactobacillus acidophilus	LG04	2	MN796080.1	90.66
Lactobacillus casei	GK21	2	KP763901.1	87.54
Lactobacillus gasseri	KL06	1	MW463614.1	93.01
Total		**10**		

*KJ= Kuje; GS=Gosa; LK= Lokogoma; GR-Giri; LG=Lugbe; GK= Garki; KL= Kwali

DISCUSSION

As quite a number of people that consume traditionally fermented drinks are unaware of the presence of the microorganisms involved in the fermentation and by extension the beneficial effects attached to their consumption, a combination of the phenotypic and biochemical properties for the identification of LAB was carried out in this research. Several studies have shown that locally fermented food products are repositories of beneficial lactic acid bacteria which are most often responsible for the overall fermentation process (Aspri *et al*, 2020) and development of industrial microorganisms (*et al*., 2014). The result of the present study has revealed that a diversity of LAB including *Lactobacillus bulgaricus* and *Streptococcus thermophilus* the two major bacteria that converts milk sugar (lactose) to lactic acid during yoghurt production (Aleksandrzak *et al*. 2015), could be found in abundance in whey, which is also a by-product of *kindirmo* processing. The LAB in the whey of *kindirmo* was isolated (using MRS and M-17 culture media), by following conventional phenotypic and biochemical tests to determine their growth pattern. The results showed that these culture media are potent selective bio-resources for obtaining pure cultures of LAB strains from mixed/wild environment which is in accordance with the work of Akabanda *et. al*. (2010).

An in vitro assay of the carbohydrate fermentative spectra of the isolates was also carried out to determine the abilities of the organisms to utilize different sugars (in this case twelve of them) viz-a-viz their homofermentative and/or heterofermentative status. All the organisms were observed to have utilized arabinose, dextrose, lactose and sucrose which is similar to the research done by Dowarah *et al.* (2018) where all LAB isolates showed a positive reaction for the fermentation and utilization of lactose, galactose, trehalose, and mellobiose This underscores the fact that the pattern of fermentation which is an indication of the characteristics of certain species/genera of organisms has been extensively used as a method for the biochemical differentiation of microorganisms (Dowarah *et al*. 2018). The sequenced products which were subjected to BLAST examination at the NCBI gene repository was a result of the PCR experiments and examination of the selected isolates. Three out of the ten isolates did not however give the expected blast results while some of the isolates (e.g., *Streptococcus thermophilus*) appeared more than once. In addition, three of the organisms appeared twice from the samples after the Consequently, the percentage identity to related *Lactobacillus bulgaricus* or *Streptococcus thermophilus* bacterial sequences in the database was 87-99%, This relatedness supports the identity of the bacterium which is consistent with the results of Bostan *et al*. (2017). This eventual confirmation of the identity of the isolates with molecular tools finally established the presence of LAB. The potential use of our strains as starter culture will depend on further studies on their probiotic potential and assessment on the effect of the quality of yoghurt.

Conclusion

This study has revealed that whey, which was hitherto a discarded byproduct of the dairy industry, could be a good source of a number of organisms such as *Lactobacillus* and *Streptococcus* species, which can play the probiotic roles in consumers. The study has proved that each strain had its individual uniqueness in terms of their ability to grow in and/or affinity for varying degrees of temperature, acidity, salt concentration and carbohydrate fermentation, which made their identification easy. The use of molecular methods in this work for the identification of Lactic Acid Bacteria (LAB) strains using the 16SrRNA gene has proven to be a veritable tool for the specific characterization and detection of LAB strains (Tamura *et al*., 2021). The 16SrRNA gene amplification revealed that the isolates were all bacteria corroborating the concept that locally fermented food products and/or ingredients thereof are an excellent natural source of beneficial bacteria. Some of the strains identified through this method have been reported to be efficient in the production of fermented milk products (Aymerich *et a*l., 2003). Hence some of the isolates can be further investigated for probiotic potential for functional food development as well as suitability for use as starter cultures for yoghurt production. This study therefore recommends that, the production process of the yoghurt drink, *kindirmo*, whose local producers have no clue as to the microbial existence and processes going on in the product, be scaled up with the use of authentic LAB strains for the fermentation which will result in a high-quality product having the desired prominence thereby making it an internationally accepted product.

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Conflict of Interest

The authors have declared no conflict of interest regarding this work.

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