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Strain-level Identification of Beneficial *Lactobacilli* of Dairy Origin using 16S rRNA Sequencing: A Biotechnology Approach

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Authors' contributions

This work was carried out in collaboration among all authors. Author SA designed the study and wrote the first draft of the manuscript. Authors COF and ABA managed the discussion and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This study investigated the cultural method and 16S rRNA gene analysis to reveal the composition and diversity of lactic acid bacteria (LAB) from dairy origin (spontaneous fermented cow milk) in Nigeria.
Methods: Six dairy samples which includes two raw cow milk, two raw goat milk and two fermented cow milk (*nono*) were collected and subjected to standard microbiological investigation using both cultural and molecular methods. The dairy samples were cultured on MRS media, and the isolates were identified using physiological and biochemical parameters. DNA of four selected probiotic LAB isolates from *nono* were amplified using PCR while the amplicons were electrophoresed in agarose gel, pre-stained with ethidium bromide and characterized by 16S rRNA gene analysis. The result of the DNA sequencing were analyzed using NCBI BLAST.
Results: A total of 55 presumptive LAB were isolated. Twenty nine (29) *Lactobacillus plantarum* representing 52.7%, *Pediococcus acidilactici* 15(27.2%), *Lactobacillus casei* and *Lactobacillus*

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brevis 4(7.3%) while *Lactobacillus fermentum* 3(5.5%) were recorded respectively. Result of the gel electrophoresis revealed DNA size of approximately 1500bp. The selected probiotic LAB from *nono* used in this study were confirmed as *Lactobacillus plantarum* N17, *Lactobacillus plantarum* N24, *Lactobacillus brevis* N10 and *Lactobacillus casei* N1 based on 16S rRNA gene sequence analysis while the phylogenetic analysis revealed a 98-100% similarity with a high homology level which affirms the strain of the organism.

Conclusion: This study has demonstrated the diversity of LAB existing in dairy samples known as fermented cow milk (*nono*) which could be harnessed as valuable sources for LAB isolation and potential probiotic organisms.

Keywords: Lactobacillus species; gene sequence; nono; fermented cow milk.

1. INTRODUCTION

The presence of lactic acid bacteria in product rich in vitamins, protein breakdown products, carbohydrate and low oxygen environment has been attributed to their complex nutritional requirements. Thus, their prevalence in dairy product [1]. Lactic Acid Bacteria (LAB) are anaerobic microbes, Gram positive cocci or rod, acid tolerant, and generally non-sporulating. They produces lactic acid as a major by-product of carbohydrate fermentative metabolism [2,3]. The lactic acid bacteria (LAB) are industrially important group of probiotic organisms that played an essential role in the manufacture and preservation of numerous fermented food products as well as in human health by inhibiting the growth of some harmful bacterial pathogens, help in eliciting immunological response, and increasing the host resistance to infections [4,5,6]. The production of assorted metabolites by lactic acid bacteria not only have pharmacokinetic properties against other microbes occupying the same ecological niche but also confer flavours in foods as well as its use in brewery industries [7,8]. Protein breakdown, fat hydrolysis and lactose metabolism are very important processes for production of high value dairy products [9]. Microbiota such as but not limited to *Lactococcus spp*, *Pediococcus spp*, *Enterococcus spp, Oenococcus spp,* and *Leuconostoc spp* are the commonly isolated organisms from raw and fermented milk [10,11,12,13]. However, the variation in the stability and quality of traditional fermented cow milk (*nono)* a spontaneously fermented cow milk (yoghurt-like) product from Northern Nigeria, Ghana, and other West African Countries is dependent on the diversity of unidentified microbial population during fermentation as no specific or identified starter culture is used [14]. The genetic diversity of the natural bacterial population of this traditional dairy product has demanded the need for improvement of useful properties of starter culture. Several molecular techniques has been used for the detection and characterisation of lactic acid bacteria or LAB with probiotic potentials from dairy product with good discriminatory capacity such as Randomly amplified polymorphic DNA (RAPD), and Amplified ribosomal DNA restriction analysis (ARDRA) [15]. More so, 16 S rRNA gene sequence analysis has been reported as the commonest and fastest molecular typing method because of clear demonstration of variation in the genome of organisms which is based on the amount of similarity of sequence between individuals strain [5]. Based of the above, this study was undertaking with a view of isolating and characterizing LAB from raw and traditional fermented cow milk (*Nono)* using primer pair previously published, to yield 16S rRNA gene fragment for identification of lactic acid bacteria.

2. MATERIALS AND METHODS

2.1 Sample Collection

Raw milk from goat and white fulani cow was procured from Dairy and Research Farm, University of Ibadan, and *Nono* (traditionally fermented cow milk) from local market in Ibadan, South-Western Nigeria. It was brought into Central Laboratory, at the University of Ibadan in sterile bottles for onward microbiological assessment.

2.2 Isolation and Purification of Lactic Acid Bacteria

Isolation of lactic acid bacteria was done using pour plate technique. One millilitre of each dairy samples from raw goat milk, raw cow milk, and *nono* (fermented cow milk) were taken aseptically and transferred into separate bottles containing 9.0 mL of sterile distilled water, and

serial dilutions of the dairy samples were made. One millilitre of 10^{-6} dilutions of the samples were inoculated on de Man, Rogosa, and Sharpe agar (MRS; Difco Laboratories, Detroit, MI) incubated in anaerobic jar at 37°C for 24-48 hours. The LAB Isolates were sub-cultured and repeated streaking was done to obtain pure culture. Typical *Lactobacillus* colonies were identified and picked for Gram staining by standard methods. The identification work was done according to the methods described in Sharpe (1981). Moreover, pure cultures of isolates were made on slants of de Man, Rogosa, and Sharpe agar, incubated at 37° C for 48 hours and stored in the refrigerator at 4°C as stock cultures of presumptive LAB for further tests.

2.3 Phenotypic and Probiotic Characterization of the Isolates

Presumptive LAB colonies were phenotypically confirmed by both physiological and biochemical properties which includes catalase test, oxidase test, starch hydrolysis, casein hydrolysis, methyl red, motility test, Vogues Proskauer test, nitrate reduction test, growth on 4% salt concentration, growth at pH4.5 and 9.6, ability to ferment maltose, ribose, sorbitol and Raffinose in MRS broth without dextrose (Difco Laboratories, Detroit, MI). The presence of arginine was investigated in MRS broth with 0.3% L-arginine using Nessler's reagents (SIGMA, France) as described by [16].

Moreover, four previously screened probiotic starter cultures such as *Lactobacillus plantarum*N24, *Lactobacillus plantarum*N17, *Lactobacillus brevis*N10 and *Lactobacillus casei*N1 [17] were subjected to molecular analysis.

2.4 Molecular Characterization of LAB Isolates

2.4.1 DNA extraction

LAB were grown in de Man Rogosa, and Sharpe broth (MRS; Difco Laboratories, Detroit, MI) overnight, 2 mL of the suspension (up to 2×10^6 bacterial cells) was centrifuged for 10 min at 5,000 ×g, and cell pellets were used for DNA extraction. DNA extraction was done using a GeneJET Genomic DNA Purification Kit (Thermo-Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

2.4.2 Polymerase Chain Reaction (PCR)

PCR amplification of DNA fragments encoding 16S rRNA was done in a final volume of 65 µl (10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 0.3units of Taq DNA polymerase (Promega, USA), 1 µl of 10 pmol each 27F-5'-AGAGTTTGATCMTGGCTCAG-3' and - 1525R-5′-AAGGAGGTGATCCAGCC-3′ primers, [18] made up to 42 µl with sterile distilled water and 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with an initial denaturation at 94°C for 5 min; followed by a 30 cycles consisting of 94°C for 30 seconds, 50°C for 60 seconds and 72°C for 90 seconds ; and a final termination at 72°C for 10 mins. The amplicon were separated by gel electrophoresis on 1.5% agarose pre-stained with ethidium bromide (0.5µg/ml), the DNAs integrity were visualized on a high-performance ultraviolet transilluminator (UVP, UK) and photographed. Molecular weights of the DNA were calculated based on the 100bp DNA ladder plus Hind III digested λ DNA used (Inqaba Biotech, South Africa).

2.4.3 Purification of amplicon

The amplicon was transferred into labeled 1.5-mL Eppendorf tubes. 60 µL of 20% (w/v) PEG 8000, 2.5 *M* sodium chloride was added to each tube and mix. The mixture was Incubate for 30 minutes at room temperature. The amplicon was pelleted and washed by adding 0.5 mL of 70% ethanol and spun at maximum speed for a further 5 min and the pellets was dried.

2.4.4 Sequence alignment and phylogenetic analysis

The purified amplicons were sequenced in a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using Big-Dye terminator v3.1 cycle sequencing kit according to the protocol established by the manufacturer. Bio-Edit software and MEGA 6 were used for all genetic analysis. Similarity searched was performed against the GenBank database of the National Centre for Biotechnology Information (https://www.ncbi.nlm.nih.gov), using the BLAST program (Basic Local Alignment Search Tool). The 16S rRNA sequences obtained were added to publicly available bacterial 16S rRNA sequences, the sequences were integrated into the database with the automatic alignment tool. The phylogenetic analyses were performed with

the Molecular Evolutionary Genetics Analysis software (MEGA 6) [19]. Evolutionary relationships among sequences were used to construct dendrograms inferred by using the sequential hierarchical and nested clustering neighbour joining method with NTSYSpc (version 2.10, Exeter Software). The robustness of the NJ topology was evaluated by bootstrap test using 500 replicates.

3. RESULTS

Six dairy samples comprising of two raw goat milk, two raw cow milk and two spontaneous fermented cow milk (*Nono*) were subjected to microbiological investigation. Fifty five presumptive LAB were isolated and identified using several physiological and biochemical parameters. Twenty nine (29) *Lactobacillus plantarum* representing 52.7% of the total LAB was the most encountered as shown in Table 1. Others includes *Pediococcus acidilactici* 15(27.2%), *Lactobacillus casei* and *Lactobacillus brevis* had 4 isolates each representing 7.3% while *Lactobacillus fermentum* the least encountered had 3 isolates representing (5.5%). The percentage of occurrence of all the isolates according to the source of the isolates are represented in Table 1. Four LAB isolates were selected randomly, and are from spontaneous fermented cow milk (*Nono*) which include *Lactobacillus plantarum*N24, *Lactobacillus plantarum*N17, *Lactobacillus brevis*N10 and *Lactobacillus casei*N1. They were confirmed by PCR and nucleotide sequencing 16S rRNA gene and, compared with available sequences in GenBank database using the BLAST program at NCBI. The results confirmed that all strains belonged to *Lactobacillus* genera. The rRNA gene sequence of the isolated LAB showed a similarity of between 98-100% identity as observed in all selected LAB, and representative isolates in the gene bank after blasting as indicated in Table 2. More so, the phylogenetic tree showed a close relationship between the LAB and related organisms except for *Bacillus subtilis* which was out grouped from the tree as shown in Fig. 2.

4. DISCUSSION

In recent times, LAB has been proposed as the most common bacteria with probiotic potentials and an integral ingredient of starters used in dairy product, hence, an upsurge in its cultivation and characterisation especially in traditionally fermented dairy products [4]. The Nigerian Northern nomadic ethnic group has developed and maintained this spontaneous fermented cow milk (*Nono*) from one generation to another. However, these nomadic group are ignorant of the starter culture used in the fermentation processes as old vessel with previous productions are used to jump start new fermentation process. In this study, we investigated and characterized LAB microflora isolated from different diary product with a view to determining the genomic properties of LAB from raw cow milk, raw goat milk and especially from traditional fermented cow milk using cultural methods. The LAB from the fermented cow milk (*Nono*) was selected and characterized by 16S rRNA gene sequence analysis. The study revealed 55 LAB randomly selected from two genera *Pediococcus* and *Lactobacillus.* The distribution of the isolates were; *Pediococcus* 15(27.2%) and *Lactobacillus* 40(72.8) from all the samples collected. Five species of LAB were recovered, this are *Pediococcus acidilactici* 15 (27.2%), *Lactobacillus plantarum* 29(52.7*%), Lactobacillus fermentum* 3(5.5*%), Lactobacillus casei* 4 (7.3%) and *Lactobacillus brevis* 4 (7.3%) respectively. Five *P. acidilactici* were recovered from raw cow milk, 10 from raw goat milk and non from *nono* (fermented cow milk). The investigation also revealed the presence of twelve *L. plantarum* from raw cow milk, two from raw goat milk and 15 from *Nono* as the predominant LAB. Three *L. fermentum*, four *L. casei* and four *L. brevis* were also isolated from *nono* only. In a report by [14], the prevalence of *Lactobacillus* from *nono* in Ghana was 53.52% as against the 72.8% recorded in this study. The difference in the prevalence rate was attributed to the sample size and the number of LAB microflora isolated, as higher number of sample was collected, and four bacteria genera was isolated in the other study as against 2 recovered in this study. In a similar study conducted in Nigeria by [20], the total percentage of occurrence of *Lactobacillus* in dairy samples was 75% which is in agreement with the 72.8% recorded in this study as the difference is considered insignificant. However, the distribution of the lactobacillus species were *L. brevis* 7.3%, *L. plantarum* 52.9%, *L. casei* 7.3% and *L. fermentum* 5.5% in this study as against *L. brevis*15.4%, *L. plantarum* 57.7%, *L casei* 15.4%, and *L. fermentum*11.5% in in other study. Despite the agreement in the percentage of occurrence of the genus *Lactobacillus* in *nono* from both studies, the disparity in the distribution of the species was as a result of the higher number of *lactobacillus* species recorded in the

other study. Four species of *Lactobacillu*s were isolated in this study while 12 species was recorded from other study. The probiotic potentials of these LAB has been previously published [17]. The selected probiotic LAB used in this study were confirmed as *Lactobacillus plantarum*N17, *Lactobacillus plantarumN24*, *Lactobacillus brevis*N10 and *Lactobacillus casei* N1 based on 16S rRNA gene sequence analysis. 16S rRNA gene sequence analysis has been reported to use the 16S rRNA gene from the isolate and explore the similarity of those that already exist in the GenBank [21]. Bacterial DNA was extracted and amplified using polymerase chain reaction (PCR). The PCR was conducted using degenerated universal primers previously published to obtain copies of 16 S rRNA gene for sequencing. The amplicons with an approximate 1500bp were electrophoresed. The results obtain from the sequencing were aligned using Bio-Edit and MEGA 6 software. The evolutionary history of *Lactobacillus plantarum*N17, *Lactobacillus plantarumN24*, *Lactobacillus brevis*N10 and *Lactobacillus casei* N1 were inferred by neighbour joining methods [22]. The major nodes in the phylogenetic tree are well-supported by high bootstrap values, which is an indication that intergroup relationships were accurately reflected. The clustering of organism within the same taxa in a phylogenetic tree has been described by [23] to produce a clear cut bootstrap value. The strains of Bacillus subtilis group was considered as outgroups, in order to root the tree. The result of the DNA sequencing was analyzed using BLAST. The 16S rRNA gene sequencing identified *Lactobacillus plantarum*N17 and *Lactobacillus plantarumN24* to belong to the same cluster while isolate N10 belongs to *L. brevis* cluster and isolate N1 to *L. casei* cluster with percentage similarity of 99% for *Lactobacillus plantarum*N17 and *Lactobacillus plantarum*N24. The percentage similarity *for L. brevis* was 100% and 98% similarity for *L. casei*. Phylogenetic tree was constructed to determine the kinship relationship of *Lactobacillus plantarum*N17, *Lactobacillus plantarum*N24, *Lactobacillus brevis*N10 and *Lactobacillus casei* N1. The study revealed 99% similarity of *Lactobacillus plantarum*N17 at 100% query coverage as same species with strain *Lactobacillus plantarum*N24. *Lactobacillus brevis*N1 showed 100% similarity at 100% query coverage for *L. brevis* (365799371) and was considered same species. 99% at 100% query coverage in 16S rRNA gene sequence analysis has been documented to having a high homology level which affirms the strain of the organism [24].

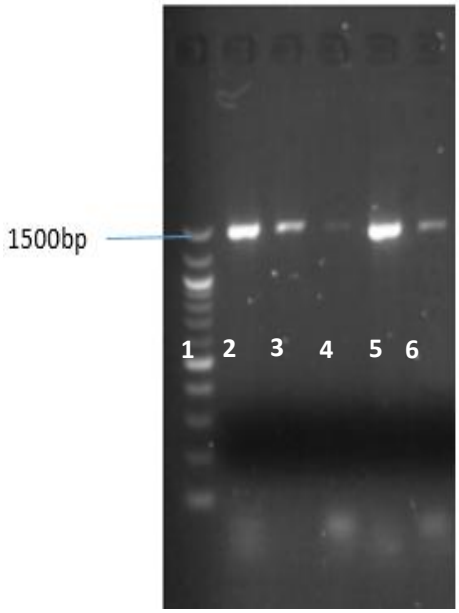

Fig. 1. Profiles obtained from representative *Lactobacillus species* isolated from traditional fermented cow milk (*nono)* samples using 1.5% agarose gel etectrophoresis. Molecular weight of ladder (1500 bp)

1= DNA ladder, 2 = Lactobacillus plantarumN24, 3=Lactobacillus plantarumN17, 4= Lactobacillus caseiN1, 5= Lactobacillus brevisN10, 6=known culture of Lactobacillus bulgaricus (positive control)

Table 1. Distribution and percentage of occurrence of Lactic acid bacteria (LAB) isolated from dairy samples

Type of sample	Number of sample	Number of isolates	LAB Isolates				
			***Pediococcus acidiolactici* (15)**	***Lactobacillus plantarum***	***Lactobacillus fermentum***	***Lactobacillus casei***	***Lactobacillus brevis***
Raw cow milk	2	17	(5) C1,C2,C11,16,C17	(12) C3,C4,C5,C6,C7,C8,C9,C10,C12, C13,C14,C15	0	0	0
Raw goat milk	2	12	(10) G1,G2,G3,G4,G5,G6,G8,G 9,G10,G11	(2) G7,G12	0	0	0
Nono (fermented cow milk)	2	26	0(0)	(15) N2,N3,N4,N6,N7,N11,N14,N16, N17,N19,N20,N23,N24,N25,N26	(3) N5,N13, N18	(4) N1,N9, N15, N22	(4) N8,N10, N12,N21
Total	6	55	15(27.2 %)*	29(52.7%)	3(5.5%)	4(7.3%)	4(7.3%)

Percentage occurrence of Lactobacillus species=72.8% (52.7+5.5+7.3+7.3)
Percentage occurrence of Pediococcus species=27.2%

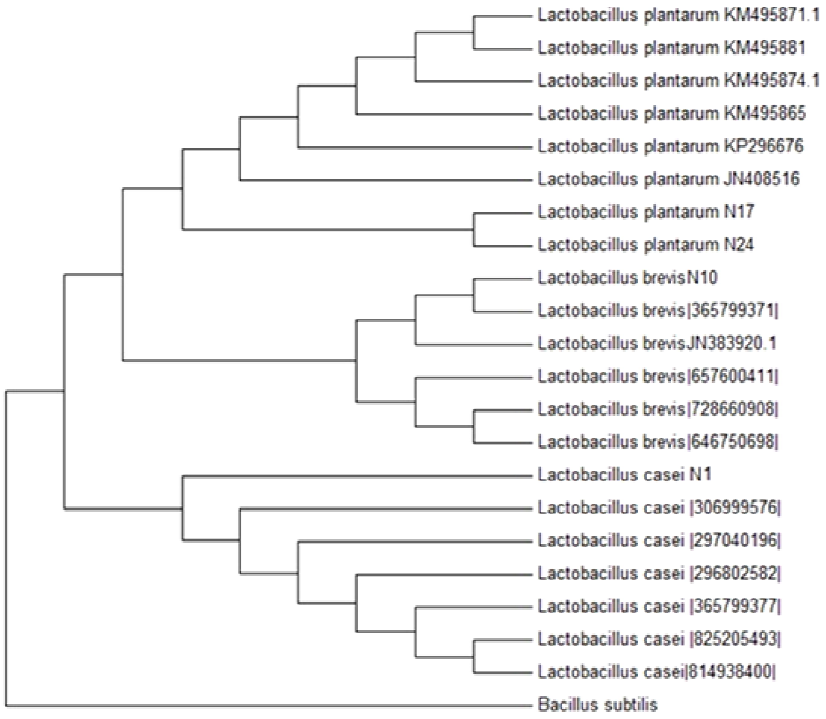

Fig. 2. Phylogenetic tree of *Lactobacillus plantarum*N24, *Lactobacillus plantarum*N17, *Lactobacillus brevis*N10 and *Lactobacillus casei*N1 based on 16S rRNA gene sequence analysis showing the phylogenetic placement of representative strains

Table 2. Molecular identification of selected probiotic LAB from fermented cow milk (*Nono*)

LAB isolates and code	Percentage similarity	Identification
*Lactobacillus plantarum*N17	99	*Lactobacillus plantarum*
*Lactobacillus plantarum*N24	99	*Lactobacillus plantarum*
*Lactobacillus brevis*N10	100	*Lactobacillus brevis*
*Lactobacillus casei*N1	98	*Lactobacillus casei*

5. CONCLUSION

The study revealed the presence of four lactic acid bacteria such as *Lactobacillus plantarum*N24, *Lactobacillus plantarum*N17, *Lactobacillus brevis*N10 and *Lactobacillus casei*N1 from traditional fermented cow milk (*Nono*) as the predominant LAB population. The diversity of Lactobacillus species found in *nono*, confirms the traditional fermented products are natural rich source of Lactobacillus strains. The PCR amplicon of the 16S rRNA gene gave an approximately 1500bp. The phylogenetic analysis also showed 98% to 100% similarity indicating high level of homology, and also as a confirmatory test. The use of larger number of samples to explore the biodiversity of microflora in *nono* from different sources in Nigeria could promote the identification of other lactic acid bacteria strains. Such lactic acid bacteria can be recommended as probiotics since genomic analysis confirmed their identity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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