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Research Article
Molecular Investigation of Potential Lactic Acid Bacteria Starter Culture Organisms/Probiotics in the Kenyan Spontaneously Fermented Milk, *amabere amaruranu*

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Abstract

Objective: The study aimed to characterize lactic acid bacteria (LAB) in Kenyan spontaneously fermented milk, *Amabere amaruranu* for identification of starter cultures. **Materials and Methods:** Twenty four samples were collected from Kisii County, Kenya and cultured on MRS agar to isolate LAB. Thirty seven isolates were selected for phenotypic (Gram staining and catalase test) and physiological [growth at 15 and 45°C, on 4 and 6.5% (w/v) NaCl] characterization and identification by 16S rRNA gene sequencing. The obtained sequences were compared to DNA sequences in the GenBank by the BLASTN program on NCBI website. The sequences were used to generate phylogenetic tree on MEGA 6.0 software. Lactic acid production and pH change capability in milk were determined as technological characteristics of the isolates. **Results:** All isolates were Gram positive and catalase negative with shapes of rods or cocci. The isolates clustered into three groups; (1) those didn't grow at 45°C but grew at 15°C 24 were considered mesophilic *Lactobacilli*, (2) One cocci that grew at 15°C but not 45°C and 6.5% (w/v) NaCl was considered mesophilic *Lactococci* and (3) Those 7 which grew at 45°C, 15°C and 6.5% NaCl, were considered *Enterococci*. DNA sequencing identified 5 isolates; two *Lactobacillus plantarum* spp., two *Epicoccum* spp. and one (1) *Staphylococcus warneri* spp. *L. plantarum* spp. coagulated milk and attained 0.73% titratable acidity and pH of 4.48 in 24 h. **Conclusion:** *Lactobacillus plantarum* spp. was identified from *Amabere amaruranu* and its technological characteristics demonstrated, hence it could be used as a starter culture for product development.

Key words: *Lactobacillus plantarum*, starter cultures, lactic acid bacteria, traditional fermented foods, *Amabere amaruranu*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fermentation is one of the oldest forms of food processing and preservation that is entrenched in many traditional cultures and village life[1,2]. Fermentation imparts desirable aroma and flavors to foods, decreases product toxicity and generates product diversity in diets including staple foodstuffs such as milk, tubers, cereals and fish[3]. Fermentation also reduces bulkiness of materials for ease of transportation, improves nutritive value and appearance of food and it reduces the energy required for cooking[4]. The importance of food fermentation is reflected by the amount of fermented foods that are traded and consumed as 20-40% of the global food supply and approximately one third of the food consumed in the current food systems is fermented food[5,6]. This renders fermented foods a substantial component of the global diet to enhance food security and improve livelihoods especially in poor rural areas in developing countries by income generation in small-scale farms.

In Africa, a number of traditional fermented products such as non-alcoholic beverages, alcoholic beverages, breads, pancakes, porridges, cheeses and milks have been documented[7,8]. Production of these foods relies on indigenous knowledge and they are informally produced at household or at small-enterprise scale[9]. In Kenya, a number of spontaneously fermented foods and beverages have been recorded[10]. Milk fermentation is a common method for preservation and a number of communities in Kenya use traditionally fermented milk. The Kalenjin community use *Mursik*, the Northern Kenya's pastoralists use fermented camel milk called *Suusa*, the Maasai community, *Kule naoto* and the Kisii, *amabere amaruranu*[11,12]. These communities have consumed these products for a long time because of their attributes of taste, flavour, consistency, colour and shelf life.

Amabere amaruranu is a fermented milk product prepared by spontaneous fermentation of milk using a gourd made from the hollowed-out fruit of *Lagenaria* spp. It is made from cow's milk that is heated to boil. The milk is then added to a small portion of fermented milk from a previous batch (*enduranerio*) after cooling and left to ferment at ambient temperature (back slopping)[10]. The milk is white in color, is lumpy in nature and acidic in taste[13]. *Amabere amaruranu* plays a central role towards the socio-economics of people in the locality through enhanced food security and income generation via micro- and small-scale enterprises.

Studies have demonstrated the therapeutic potential of *amabere amaruranu*. For example, Boyiri[14] identified *Lactobacillus rhamnosus* strain in *Amabere amaruranu* that could stimulate increase in MUC4 and MUC3 expression in colon cells. Kotala and Onyango[15] also demonstrated that the cell extract of *L. rhamnosus* from *amabere amaruranu* downregulates the expression of numerous adipogenic-related transcription factors and at high doses, the cell extracts downregulated the peroxisome proliferator-activated receptor-ɣ, sterol regulatory element-binding protein 1 and adipose triglyceride lipase. Mokua[16] also demonstrated the antibacterial effect of *Amabere amaruranu* on *E. coli*.

Despite the potential of *Amabere amaruranu* as a source of probiotics, its poor hygiene and post-fermentation handling limits its shelf-life[10]. Moreover, regulatory bodies will only accept information on health claims of this product if it is backed by scientific evidence, which could only be possible with big industrial players[17]. Fermented foods such as *amabere amaruranu* could be recommended for improving the health and nutritional quality of traditional African foods and regular inclusion of fermented products as part of the daily diet would be desirable[5] . However, lack of knowledge and understanding toward *amabere amaruranu* preparation may limit its usage.

Studies have shown that some fermented foods in Africa may pose a safety risk[18]. These safety issues are demonstrated by the deaths and risks of esophageal cancer reported by the consumption of fermented milk products from Kenya including *Mursik*[19-21]. The technology for the production of *amabere amaruranu* has not been upgraded into industrial scale to meet the growing demand for traditional fermented products by the urban and immigrant population. The household production is laborious and time-consuming and the starter culture are absent; hence the need for production of *amabere amaruranu* efficiently using starter cultures with assured safety, quality, packaged for extended shelf life, broader acceptance and in a ready-to-use form from high quality milk[10]. This could only be possible if the microbes in the *amabere amaruranu* are characterized and starter cultures isolated.

Indigenous fermented milk products contain microbiota composed of technologically important species and strains which are gradually getting lost with new technologies[13]. In making these products, microbes in raw milk and the processing environment serves as inoculants for fermenting the milk. The fermentative microbes enhance properties such as taste, aroma, shelf-life, safety, texture and nutritional value of foods[9]. Some microorganisms present in traditionally fermented milk products have been documented in various studies[11,12,22-25]. The most predominant lactic acid bacteria (LAB) reported includes *Lactobacillus fermentum*[23], *Lactobacillus plantarum*[12], *Leuconostoc mesenteroides*[11]

and *Streptococcus thermophilus*[24]. The most commonly encountered yeast genera include *Saccharomyces* spp., *Candida* spp. and *Trichosporon* spp.[11,23].

Isolation and screening of microorganisms from naturally occurring processes is the most powerful means for obtaining useful cultures for scientific and commercial purposes[9]. The diverse mixture of LAB and yeasts microbiota forms a potential consortium for further product innovation in *amabere amaruranu* and other fermented milk products. A previous study on the *amabere amaruranu* using culturing techniques identified some LAB and yeasts but failed to identify 40% of the yeasts[13]. Despite *amabere amaruranu* being there for many generations, few studies have been done to isolate and identify the microorganisms involved in its fermentation and starter cultures have not been developed for its production[26]. According to Holzapfel[4], there is an increasing need to select microbial strains with functional properties for commercial production and for improvement of quality and safety of existing traditional fermented food products. The aim of this study was therefore to use molecular techniques to characterize potential starter cultures (Lactic acid bacteria) from *amabere amaruranu* and demonstrate their potential for product development.

MATERIALS AND METHODS

Sample collection: *Amabere amaruranu* samples 24 were purposively collected from households at Mosocho center in Kisii County, Kenya. At least 200 mL of samples were picked, put in sterile and labeled plastic containers with tight fitting caps. The samples were cooled immediately by putting them on ice blocks in cooler boxes and transported to the laboratory. Samples were analyzed immediately and those not analyzed were refrigerated for later analysis.

Enumeration and isolation of lactic acid bacteria: Ten milliliters of each sample was homogenized in 90 mL sterile diluent [1% peptone (Difco, Detroit, Michigan, USA), 0.85% NaCl, pH 7.0] using a stomacher (Stomacher- Bagmixer, Buch and Holm) for 30 sec. Tenfold serial dilutions (10^{-1} to 10^{-9}) were made with the same diluent and 0.1 mL aliquot of each appropriate dilutions was spread-plated while 1 mL was pour-plated in duplicates on de Man, Rogosa and Sharpe (MRS) agar (Oxoid Ltd, Basingstoke, Hampshire, England), incubated at 35°C for 2 days anaerobically using the Anaerocult A pack (Merck, Darmstadt, Germany). Discrete colonies from pour plates of the highest dilution of MRS agar were selected and isolated based on their shape, size, colour and gloss. The isolated colonies were purified by repetitive streaking on MRS media and stored in 0.25 mol L^{-1} sucrose solution at -18°C until required for identification.

Phenotypic and physiological characterization of lactic acid bacteria: Colonies on individual culture plates were examined for shape, size, elevation, surface characteristics and edges then the Gram stain andca talase tests were used to characterize the isolates. Growth at 15 and 45°C was determined by culturing the isolates on MRS broth and observing visually for turbidity after 72 h of incubation. Gas production from glucose was determined according to the methods described by Harrigan[27], using MRS broth as basal medium. Salt tolerance test was done using MRS broth containing 6.5 and 4% (w/v) NaCl. Tubes were then inoculated and incubated for 4 days at 37°C. A positive result was detected by visual inspection for an increase in the turbidity of the solution.

Molecular characterization of lactic acid bacteria

Genomic DNA isolation and sequencing: Genomic DNA was extracted by the CTAB DNA extraction procedure described by Cardinal *et al.*[28]. The extracted DNA was tested for quantity and quality using Nano drop photometer and gel electrophoresis. Dissolved genomic DNA samples were then stored at -20°C. Samples of dissolved DNA were sent to Inqaba biotechnical industries Ltd, Pretoria, South Africa, for 16S rRNA partial gene sequencing using group-specific primer pairs Lac1 (5'AGCAGTAGGGAATCTTCCA') and Lac2-GC (5'GATTYCACCGCTACACATG'3) to detect the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella*[29] and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGC TTATTGATAT GC-3') for discrimination of fungal species based on the internal transcribed sequence (ITS) (5.8S rRNA-ITS gene regions of the fungal isolates)[30]. The 16S rRNA gene targeted primers pair 907R© (5'CCGTCAATTCCTTT(AG)AGTTT3') and 1492R (5'GG(CT)TACCTTGTTACGACTT3') were used to analyze sequences that were undetected using Lac1 and Lac 2 and ITS1 and ITS 4 primer pairs.

Sequence data analysis: The partial rRNA gene nucleotide sequences were analyzed and determined using the BLAST algorithm[31]. Consensus sequences were imported into MEGA version 6.0 software[32], with which a sequence alignment and phylogenetic trees were created based on the neighbor-joining (NJ) method. The percentage of bootstrap confidence levels for internal branches, as defined by the MEGA program, was calculated from 100 random resamplings.

Technological characterization of isolates as potential starter cultures for product development: To determine acidifying activity, potentiometric (pH measurement) and titrimetric methods were applied[33]. The isolates were activated from frozen stocks in MRS broth for 24 h at 37°C and 0.1 mL

overnight cultures were inoculated in 10 mL of sterile UHT skim milk broths. Duplicate inoculations were prepared. After the 3th, 5th, 8th and 16th h incubations at 24, 37 and 45°C, 2 mL aliquots were taken aseptically and used for the procedures for determination of acidity development and pH change. Then graphs were plotted for comparison for the different isolates.

For pH determination, a pH meter with glass electrode was used (Hanna instruments, PH 211 Microprocessor PH Meter). Before using, pH meter was calibrated with buffer 1 (pH 7.0) and buffer 2 (pH 4.0). After the calibration, the glass electrode was soaked into each of the samples and pH values were recorded. To monitor lactic acid production, on to 9 mL aliquots of MRS broth media, 2-3 drops of phenol phthalein solution were added as indicator. Samples were then titrated by using standardized 0.1 N NaOH solutions and acidity calculated where each one 1 mL of 0.1 N NaOH was taken to be equal to 9.008 mg of lactic acid.

RESULTS

Characterization of lactic acid bacteria isolates: All the isolates (37) obtained on MRS agar were Gram positive and catalase negative (Table 1). For growth characteristics, 34 isolates grew at 15°C while three isolates did not grow. This was in contrast to growth characteristics at 45°C where 27 of the 37 isolates grew at this elevated temperature. With regard to growth characteristics on medium containing NaCl, over 80% of isolates grew at 4% NaCl while only 70% of the isolates grew on the media containing 6.5% NaCl. Among the isolates, 81% of them were rods, 13.5% appeared as short rods, while 5.5% appeared as cocci (Table 1).

Molecular characterization of lactic acid bacteria

DNA isolation and quantitation: Genomic DNA was extracted from the isolates cultured on 3 mL test tubes and was

Table 1: The growth of Lactic acid bacteria culture isolated in MRS media, Gram staining, catalase reaction and growth at different temperatures and salt concentration.

No.	Isolate code	Gram reaction	Catalase reaction	Growth at 15°C	Growth at 45°C	Growth at 4% NaCl	Growth at 6.5% NaCl	Cell shapes
1	D_2C	+	-	15	-	-/+	+/-	Short rods
2	DW	+	-	0	-	+	-	Short rods
3	$R_{13}SC$	+	-	0	+	+	-	Rods
4	$R_{13}SW$	+	-	0	-	-	+	Rods
5	$R_{23}C$	+	-	-	-	+	+	Rods
6	$R_{23}SRW$	+	-	0	+	-	-	Rods
7	$M_{11}W$	+	-	0	-	+	+/-	Short rods
8	$M_{21}W$	+	-	0	-	+	-	Short rods
9	$M_{22}WC$	+	-	0	-	+/-	-	Short rods
10	$M_{31}DW$	+	-	0	-	+	-	Rods
11	$M_{31}SC$	+	-	0	+	+	-	Rods
12	$B_{16}SW$	+	-	0	-	+	-	Rods
13	$B_{26}SW$	+	-	0	-	-	-	Rods
14	$B_{26}DW$	+	-	0	-	-	+	Rods
15	$B_{14}CH$	+	-	0	+	-	+	Streptococci
16	$B_{14}SW$	+	-	0	-	+	-	Rods
17	$B_{13}SW$	+	-	0	-	+	-	Cocci
18	$A4^5W$	+	-	0	-	+	+	Rods
19	$A22^5C$	+	-	0	-	+	+	Rods
20	$A17^6C$	+	-	0	-	+	+	Rods
21	$A14^6W$	+	-	0	-	+	+	Rods
22	$A9^6W$	+	-	0	-	+	+/-	Rods
23	$A21^5W$	+	-	0	-	+	+	Rods
24	$A21^5C$	+	-	0	-	+	+	Rods
25	$A10^6C$	+	-	0	+	+	+	Rods
26	$A18^6W$	+	-	0	-	+	+/-	Rods
27	$A15^6C$	+	-	0	-	+	+	Rods
28	$A13^6W$	+	-	0	+	+	+	Rods
29	$A15^6W$	+	-	0	+	-	+	Rods
30	$A9^4W$	+	-	0	-	+	+	Rods
31	$A12^5W$	+	-	-	-	+	+	Rods
32	$A10^6W$	+	-	0	-	+	+	Rods
33	$A21^6W$	+	-	-	-	+	+	Rods
34	$A17^6W$	+	-	0	-	+	+	Rods
35	$A2^6W$	+	-	0	+	+	+	Rods
36	$A3^5W$	+	-	0	+	+	+	Rods
37	$A27^6W$	+	-	0	+	+	+	Rods

electrophoresed on 0.8% agarose gel. The image of the gel was taken in Gel Doc. system (Bio-Rad, California) and intact bands (Fig. 1) were obtained after running the gel at 80 volts for an hour. The concentration and purity of the extracted genomic DNA was quantified using a Nanodrop spectrophotometer (Implen, Germany)at 260 nm. The DNA concentration ranged between 93 and 610.2 ng μL^{-1}. The 260/280 ratios of the extracted DNA ranged between 1.32-2.01.

Identification of isolates by 16S rRNA gene sequencing: Genomic DNA of 5 isolates were used templates in PCR amplifications of the V3 region of the bacterial 16S rRNA gene, using a group-specific primers (Lac 1 and 2) for the detection of members of the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella*[29]. The primer pair only amplified 3 isolates; sample number 24 ($A^6$8), 27 ($M^1$2W) and 28 ($B^6$2W) whose 16S rRNA gene sequence data is presented in Sequence data 1. Subsequently other universal primers

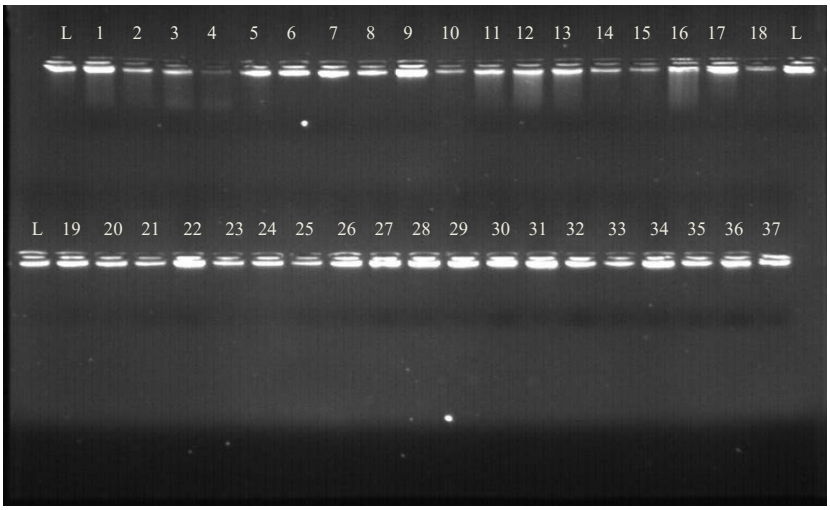

Fig. 1: DNA quantification gel image. Letter L represents standards at 50ng/ul

Sample number 24 ($A^6$8) Lac1 DNA sequence
5′TTATKGGAGCACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTG
TTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTC
CGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGA
AACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTASGGGTGRAATCA3′

Sample number 24 ($A^6$8) Lac2 DNA sequence
5′ATCAAGTTTCCAGTTTCCGATGCACTTCTTCGGTTGAGCCGARGGCTTTCACATCAGACTTAAAAAACCGCCTGCGCTCGCTTTAC
GCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATACC
GTCAATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGT
TGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCCTACTGCTA3′

Sample number 27 ($M^1$2W) Lac1 DNA sequence
5′GGKMYTARGARMAACSCCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACAYMTYTKAGAGT
AACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCAT
CGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGRAATCA3′

Sample number 27 ($M^1$2W) Lac2 DNA sequence
5′CCTTKYKRGMMWMAGTTTCCAGTTTCCGAKGCACTTCTTSGGTKGAGCCGARGGCTTTCACATCAGACTTAAAAAACCGCCTGCG
CTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGG
TTAAATACCGTCAATRCCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCA
CGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTA3′

Sequence data 1: Continue

Sample number 28 ($B^6$2W) Lac1 DNA sequence
5'CRGGRRSCAMSCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATCAGGGAAGAACAAMYGTGTAAGTAACTGTGCA
CRTCTTGACGGTACCTGATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAAT
TATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAA
AACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGRAATCA3'

Sample number 28 ($B^6$2W) Lac2 DNA sequence
5'GWWCKMCTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCG
CGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGATCA
GGTACCGTCAAGAYGTGCACAGTTACTTACACRTTTGTTCTTCCCTGATAACAGAGTTTTACGATCCGAAGACCTTCATCACTCACGCG
GCGTTGCTCCGTCAGGCTTTCGCCCATTGTGGAAGATTCCCTACTGCTA3'

Sample number 6 (R^3SC) ITS-1 DNA sequence
5'GMMAARSGRGKKGKAAACYTTCGGTCTGCTACCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCYCGCCGATT
GGACAACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAAAACATAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC
CCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCCGCGTGT
AGACTCGCCTTAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAA
AAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA3'

Sample number 9 (B2SW) ITS-4 DNA sequence
5'AGGTCMRKKYCCTCGAGGTYATKRGTGTRMWAATGTACTTKTGGACGTCGTCRKTATGAGTGCAAAGYGCGAGATGTACTGCGCTC
CGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTACACGCAGAGGCGAGACAAACACCCAACACCAAGCAGAGCTTGA
AGGTACAAATGACGCTCGAACAGGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTC
TGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTTATGT
TTTTTCAGACGCTGATTGCAACTGCAAAGGGTTTGAATGTTGTCCAATCGGCGGGCGGACCCGCCGAGGAAACGAAGGTACTCAAAA
GACATGGGTAAGAGGTAGCAGACCGAAGTCTACAAACTCTAGGTAATGATCCTTCCGCAGGTTCACCTACGGA3'

Sample number 9 (B2SW) ITS-1 DNA sequence
5'ACGGGAAGACTTCCGKAGGTGCTACCTGYKRCCCATGWYTTYTGAGKCCTWCGATTCCTCGGCGGGTCCGCCCGCCGATTGGACAA
CATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAAAACATAAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGG
TATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGTGTAGACTC
GCCTTAAAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGTAC
ATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGRAA3'

Sample number 24 ($A^6$8) 907-R DNA sequence
5'CMRGCCGGGWGTGCCTTAYGCGTTAGCTGCRACACTGAAKRASTRRAWAYACCTMCCATCTAACACACTCATCKTTACCGCGTGGA
CTACCCAGGGATCTAATCCTGGTTGCTCCCCAACCTTTCCSGCCTCARSGGYMRKTAACAGACAAGATGCCCSCCTCCCCACCTGGGG
TCTTTCCATAATTTACCAATTTCACCTCTACCCTGGGAAATCCCCCTCCCTCCTTCACACTCTARKYTGCCCRKATYMMATGGACCTTCC
CSGGTAGASCCGGGGATTTCACATTTRAATGTAMRAACCCSCCAGCGCTCCTTTACSSCCCAWYATTTCCAGCAAASSTTKGCCCCTTA
CKATTACCCGSGSTGGTGGSACSAAAKTARSCCGGGSTTTTTTGGYKGGAACCGKYMTCGGGATGACCGSCKAATCTGATTTTTGTTCTT
AAAACCTTCTTCACACACACGAYATTGCTGGATTCTGGWTTCCCCCGTTGTCCAATATTCCCCACTGCTGCCTTTGCCGGAGTCTCCCT
ACTGCTGCCTCCCCAGTGAGGCTGATCATCCTCTCAGACCARMTGTGGATSATCRCCCTGTCAGGYCATTAATCCATCRWGRMCTTGA
TGARACGYWAGCTCCTCMACTAGCTAMTGGMSCCTTTGACCMTMMRGYGKCATGMSGYATTAGCTCCTTTTTCATARAAACATTGCC
CACCCATGGATAGTATCCTATKAGYWACTGTTTCGWMGKGCACTAMSGTCSAGATGGGCRKGYKACTTGCRTGTGACTCRCMTGYYC
SCMRCTCTCGTTTTKASSGTGWAMMAAARMTCTAGTGGAAWAARGAAGCGTWCGACTYGCATGTATTAGGCACGCCGMCASCGTT
CGTCCTGAKMCATGATTYCAAACTTYT3'

Sample number 24 ($A^6$8) 1492-R DNA sequence
5'CMWGWAGGGCGGGGTGTGCTCCACTATGYKRSYTCRMYSACKTGCTKRWGGTMMAAMCTMCTCCCATGGTGYGACGGGCGGGT
GWACGSCCCCGAACAAATTATTCGCGSGTGTGGTGATCCGATTTTACTAGATATTCCMCTTTCGTGSGCTAGATGTGCACAGYGMAM
TCAA+ATCTGAGAAGGTTTTTAAAAATAASTTAGATKTCSSAATTTASCTTCCCACTGACTTCCCCTTTGAASMASKGGGGWASCCCAG
GGMATAAGGGCCATGAGGACTTGACKYMTTCCCCACCTTCCTCCGGTTTGYCACCGGMRKYYTSTCTAAAGGGCCCACCCAAASATG
STGRCWAMTAAWRATGGGGGYTGCSCTCGYTGGAGTAATTAACCCAACATCTCACRACACGAGATGACRACCRTCATGCACCTGCTG
YTTTGYCCGCCCCTGGGAAGAAATATCCWTCTATGGATACAASGAACCCATAAMCTGCCTGGGAAGGTTCTGCGGGTTGCTTWTAAA
TAAACCGCATGATCCACCGKTSGGGCGGGCCCCCATTAATTCCTTTGASWTTCTTGCTTGCGGACTTACTCCGCAGGSKGYGTGATTAA
YKMGYTGCCTGMSACACTGAAKAACTAACTTACCCAACATCTASCACACATCTTATTACAGSGKGRACTACCAGGGTATCTAATCCTGT
GYTGCTCCCCCACTTTTTCGCCGCCTCAKCGTYWAYTATRARMCARAGYCGCCTTCTTCRCCACCGGTGTTCTTCMYAATATCTACATA
WTYWYCRCCTCTACACTGARTAYWCCACWMCTCTTCTCTCACACTMTAKTCTGCAYKTMTMAWGATGCASCTCCSAKTGAKAMGSS
SSGGGTTATTTCACATCTKWMTGWAMAMMGCCGYGCTACWCGCTCTTYTACGCMCWARWYWYTCSASARMAACTTSTCGMCCCY
WCTTTMRTTATTASCGCGTGCTGSTGRCGACARAWKWTMSGCGGGKTCTTYTGTCTAGAAKRACTASCGWCRKSATGCAATCMGCTG
ACYSAWMAGTSCTTGTWYATCYGTAAAMACATGCAGTTCWYTACCRTCGTATRGCTTGCATCACGTCTGCCGCGATGCTCGATMATA
CTACTGCATCGTCGATAGTACTCTAGCGTGCCGTGCCTCYCCAGTACCGAGTTTGTGGG3'.

Sequence data 1: Amplified DNA sequences

Table 2: Genotypes of the 5 isolated microorganisms by ribosomal DNA gene sequence alignments submitted to the NCBI Gene Bank database (BLAST)

Strain No.	Strain name	Gene bank acc. No.	Query coverage (%)	Max. ident. (%)
24 ($A^6$8) Lac 1	Uncultured *Lactobacillus* spp.	JF427675.1	98	99.02
24 ($A^6$8) Lac 2	*Lactobacillus plantarum* strain LPP19	KY614058.1	99	99.34
27 ($M^1$2W) Lac1	*Lactobacillus plantarum* strain syn3	KM023152.1	94	98.33
27 ($M^1$2W) Lac2	*Lactobacillus plantarum* DCRUST PKLP4	MH548358.1	95	98.33
28 ($B^6$2W) Lac 1	*Staphylococcus warneri* strain IAE237	MK414942.1	95	98.66
28 ($B^6$2W) Lac 2	*Staphylococcus warneri* strain A28	MK712427.1	97	99.01
6 (R^3SC) ITS-1	Uncultured *Epicoccum* clone SW 2d G08	JF449836.1	96	99.80
9 (B2SW) ITS-4	*Epicoccum nigrum* isolate ECU67	MF435115.1	93	99.16
9 (B2SW) ITS-1	*Epicoccum nigrum* isolate AS-S-19	MK632017.1	96	97.36
24 ($A^6$8) 907-R	*Lactobacillus plantarum* strain UIGOA134	KY817129.1	60	73.60
24 ($A^6$8) 1492-R	No significant similarity found	-	-	-

were used and the ITS pair of ITS-1 and ITS-4 amplified 2 isolates, number 6 and 9 while the primer pair of 907R and 1492R amplified isolate number 24 only and the DNA sequences are presented in Sequence data 1.

The resultant nucleotide sequences were BLAST-searched for homology with known sequences in the NCBI database. The results in Table 2 indicated that the nucleotide sequences of the 5 strains aligned with the rRNA gene sequences of different species belonging to three genera, namely, *Lactobacillus* [24 ($A^6$8) and 27 ($M^1$2W)], *Epicoccum* [6 (R^3SC) and 9 (B2SW)] and *Staphylococcus* 28($B^6$2W) with identities of over 97%. At species level, the *Lactobacillus* was *L. plantarum*, *Epicoccum* was *E. nigrum* and for *Staphylococcus,* it was *S. warneri* although it was difficult to discern at strain level using the rRNA gene sequencing.

The partial rRNA gene sequences 11 were aligned to construct a phylogenetic tree and the phylogenetic position of these strains was then compared with related taxa in a dendrogram (Fig. 2). Sample number 24 ($A^6$8) and 27 ($M^1$2W) showed close proximity and were close to *L. plantarum* for all the sequences amplified because they grouped on the phylogenetic tree except for primer 1492-R amplicon that resulted into a sequence that did not match any sequence in the DNA database. Sample number 6 and 9 also showed close proximity and were close to *Epicoccum nigrum* and the partial sequences amplified by the same primer grouped together on the phylogenetic tree (Fig. 2). On the other hand, sample number 28 was alone in the phylogenetic tree and its partial 16S rRNA gene sequence showed high identity to *Staphylococcus warneri.* In all the cases, it was difficult to discern the isolates at strain level.

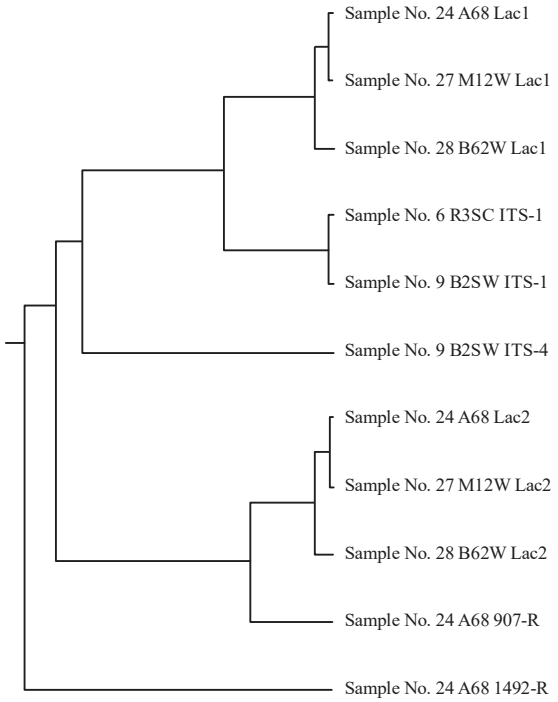

Fig. 2: Neighbor-joining tree showing the phylogenetic relationships between isolates of *Lactobacillus plantarum*, *Epicoccum nigrum* and *Staphylococcus warneri* species based on rRNA genes

Determination of the fermentation capability of lactic acid bacteria isolated for use as potential starter culture for product development: The identified isolates were analyzed for their performance as potential starter cultures. Their acid production and pH change with time were determined at 24, 37 and 45°C (Fig. 3). In the time course experiment, the results revealed that the acidifying activity (technological criteria) of *L. plantarum* isolate number 24 was significantly higher than the activity of the other species over a 16 h period and this corresponded to a faster drop in pH over the same period of time. However, the highest acidity attained at this temperature was 0.31% and a pH of 5.85. At 37°C, *L. plantarum*, strain number 24 was the fastest in acidifying activity and from 0-8 h, the rate of acid production was faster than at 24°C and from the 8th h towards the 16th h, acidifying activity was faster and reached 0.47%, which was significantly

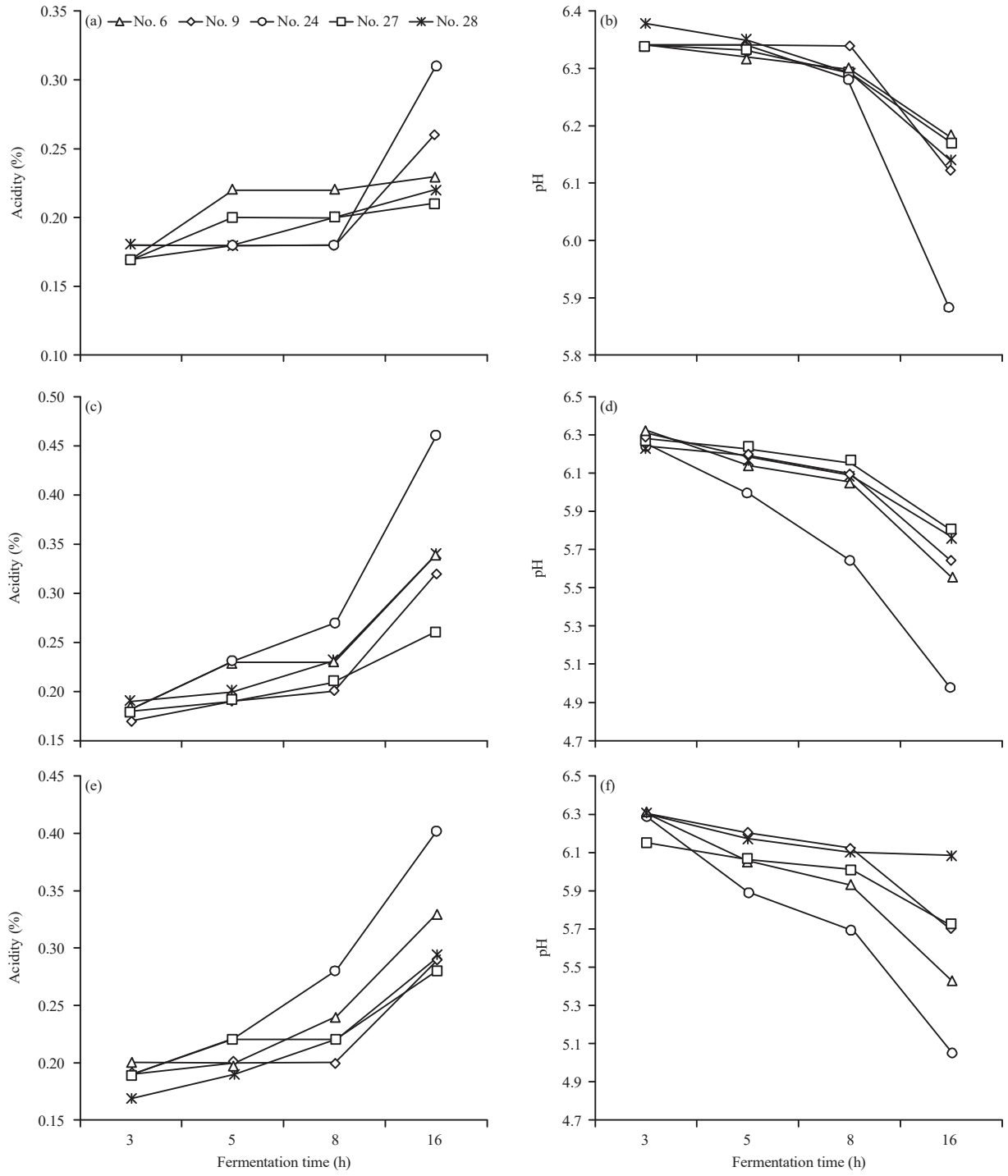

Fig. 3(a-f): Time course graphs showing acidity development (a, c and e) and pH change (b, d and f) for the different isolates at 24°C (a and b) 37°C (c and d) and 45°C (e and f)

higher than 0.31% reached at 24°C. At 37°C, the pH also dropped to 4.95 for *L. plantarum* (number 24) and was more acidic than 5.85 attained at 24°C by the same strain. *L. plantarum* (number 27) although identified as the same species as number 24, was slower in acidifying activity than number 27. At 45° C, *L. plantarum* number 24 was still the fastest in acidifying activity reaching 0.4% after 16 h with a corresponding pH of 5.05, signifying that the organism had

better acidifying activity at 37°C than at 45 and 24°C. Strain number 6 identified as *Epicoccum nigrum* was found to be second best in acidifying activity especially at 45°C. From these preliminary studies, 37°C was selected for product development using the *L. plantarum* strains (numbers 24 and 27).

Fermentation potential of the isolated strains: The two *L. plantarum* strains were cultivated at 37°C and acidifying activity evaluated at this optimum temperature over a period of 24 h (Fig. 4). It was observed that the strains were similar in their acidifying activity and were clotting the milk, characteristic of fermentation. They were both slow for the first 7 h, after which acid production was rapid up to 18 h where production slowed and it reached 0.64% for strain number 24 and 0.73% for strain number 27 after 24 h (Fig. 4a). The increases in titratable acidity corresponded to pH drop that slightly changed in the first 7 h but thereafter dropped steadily until it reached 4.48 and 4.92 for strains number 27 and 24, respectively after 24 h of cultivation (Fig. 4b).

DISCUSSION

Morphological and Physiological identification of lactic acid bacteria: All isolates were microscopically examined for Gram stain reaction, cell morphology and cellular arrangement. All the 37 isolates were Gram positive and catalase negative with cell shapes of rods or cocci, giving an implication of presumptive isolation of LAB. According to Salminen *et al.*[34], LAB are Gram positive bacteria in the shapes of rod or round and are catalase negative. Based on morphological characteristics, the isolates were divided into three groups as follows: Those isolates that grew at 15°C but didn't grow at 45°C were considered as mesophilic *Lactobacilli*[35] and 24 isolates were in this category. Majority of LAB in *amabere amaruranu* are expected to be mesophilic since the product is normally fermented at ambient temperatures where mesophiles are expected to predominate. Gram positive, catalase negative cocci, which grew at 15°C but not at 45°C and 6.5% (w/v) NaCl, were considered mesophilic *Lactococci* and one isolate fell into this category. Those isolates which grew at 45°C, 15°C and 6.5% NaCl were considered as *Enterococci* (such as isolates number 15, 25, 28, 29 and 35-37) despite their appearance as rods (Table 1). *Enterococci* are not GRAS because of their role as pathogens. However, most of the strains are safe to use in dairy fermentations, hence their presence is not alarming[36]. The presence of *Enterococci* in *amabere amaruranu* is probably due to faecal contamination of milk during milking.

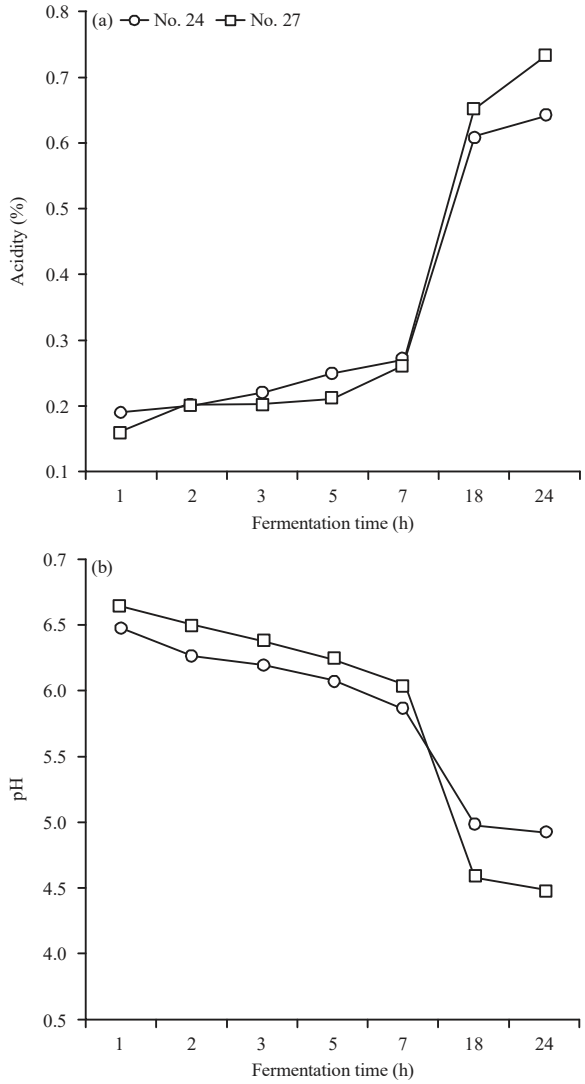

Fig. 4(a-b): Acid production (a) and pH change by the two strains of *L. plantarum* isolated from *Amabere amaruranu* at 37°C. Sample No. 24 and sample No. 27 = the two isolates used. pH 24 = Isolate No. 24 pH change curve; pH 27 = Isolate No. 27 pH change curve.

The examination of the influence of temperature was aimed to understand the type of bacteria as the result of isolation, whether it belongs to psychrophilic, mesophilic or thermophilic groups. Psychrophilic microbes grow at temperature less than 15°C and optimum at 20°C. Mesophilic group can grow at warmer temperature, ranging from 15-45°C, while thermophilic group can grow at high temperature, which is between 45 and 80°C. More than 90% of the isolates were able to grow at 15°C, 34.1% were able to grow at 45°C while 65.9% were able to grow at 6.5% salt concentration and they were considered salt tolerant. This

examination gave an indication of the osmotolerance level of the LAB strains. According to Adnan and Tan[37], high osmotolerance would be a requirement of LAB strains to be used as commercial strains, because when lactic acid is produced by the strain, alkali would be pumped into the broth to prevent an excessive reduction in pH and the free acid would be converted to its salt form, increasing the osmotic pressure on the bacteria. Therefore, most of the isolated strains were fit for use as starter culture for production purposes however, after identification.

Molecular characterization of lactic acid bacteria: The quantity of DNA was measured by the nucleic acid values while the quality of the DNA was measured by 260/280 values which the acceptable values ranging from 1.5-2.2 where pure DNA should be 1.8 while pure RNA should be 2.0. Therefore, the quantity and quality of DNA extracted from the isolates was within the acceptable limits for PCR amplification and sequencing. The partial DNA sequence for isolate No. 24 and 27 identified them as *Lactobacillus plantarum.* Isolate No. 6 and 9 were also identified as *Epicoccum* spp. while isolate number 28 had close proximity to *Staphylococcus warneri*. With the rRNA gene sequencing, it was difficult to discern the isolates at strain level, showing the limited discriminatory power of the method for identification to strain level; hence genotyping methods such as Random Amplification of Polymorphic DNA-PCR (RAPD-PCR) analysis could serve as alternatives to discriminate the isolates at the species level[38]. The isolates in this study were first cultivated on MRS media that is routinely used for the isolation and counting of LAB from most fermented food products. In Table 1, isolate No. 6 and 9 had already been designated as rods and short rods, respectively, based on morphological and physiological characteristics. However, MRS medium is known to exhibit poor selectivity for *Lactobacilli* [39], hence the detection of fungus on a *Lactobacillus*-specific MRS media.

Lactobacillus plantarum was the main LAB isolated in this study. *Lactobacillus plantarum* is a versatile species that has useful properties and is mostly found in a number of fermented food products [40]. For instance, Mathara *et al.*[17] found *L. plantarum* to be the major *Lactobacillus* species in *Kule naoto*, where it was thought to be responsible for the characteristics of the product. Moreover, the species has been reported in *Mursik*[41] and was also the most prevalent *Lactobacillus* species in *amabere amaruranu* where it was reported to comprise 20% of the total isolates[13], which implies that the species plays a vital role in the fermentation of *amabere amaruranu*. The *L. plantarum* is widely employed in industrial fermentation and processing of raw foods and is "generally recognized as safe" (GRAS) and has qualified presumption of safety (QPS)[42,43]. With the continued growth of the market for fermented food and ingredients coupled with increased consumer demand for healthy food products, the isolate will help is the design of healthy foods, because *L. plantarum* strains have been described to have known probiotic properties. Moreover, the strains have an outstanding effect on the flavor and texture in fermented foods[44]; hence ease of acceptability of *L. plantarum*-fermented food products. Therefore its further evaluation as a probiotic organism and as a starter culture is necessary.

Epicoccum nigrum is ubiquitous black yeast[45], which clearly indicates that the environment where milk is produced plays a key role in the quality of the product. Yeast and moulds survive well under low pH hence able to thrive in the spontaneously fermented milk. The interest in *Epicoccum* spp. could be in its many secondary metabolites such as polyketides, polyketide hybrids and diketopiperazines, among others, that it produces[46]. Many of these metabolites present biological activities, such as antimicrobial, antioxidant, anticancer and inhibition of viral replication, making *Epicoccum* species important producers of compounds with potential biotechnological applications. The telomerase inhibitor D8646-2-6 has been isolated from *E. nigrum*[47,48]. Moreover, *E. nigrum* produces taxol, which is an anticancer metabolite[49]. All these show that although *E. nigrum* is a contaminant in fermented milk, it could confer health benefits and has potential application as a probiotic in milk and possibly other food products.

Isolate No. 28 was identified as *Staphylococcus warneri*, which is a member of coagulase-negative *Staphylococci* (CNS). Generally, CNS species occur as skin commensals and are considered to be harmless but they often turn into opportunistic pathogens of low virulence[50]. CNS species are occasionally found in food samples, especially in fermented foods[51]. For instance, they are frequently found in fermented meats (i.e. fermented sausages), where their presence is usually considered as safe. *Staphylococcus warneri* species have also been found in French cheeses, dry fermented sausages, processing environments and clinical samples. Three strains of *Staphylococcus warneri* isolated from meat samples were found to produce warner in, peptide bacteriocin; which inhibits the growth of a large number of Gram-positive and Gram-negative bacteria, hence the strains of *S. warneri* have potential as probiotics[52]. Its presence in *amabere amaruranu* could be associated with contamination from the cows or human handlers; however, its role in the milk as a pathogen or as a probiotic needs to be investigated.

Acidifying activity of the identified isolates: All the 5 isolates that were satisfactorily identified by rRNA gene sequencing were further selected according to technological criteria. Acid production properties of LAB are the main technological characteristics for the dairy industry[53]. For this aim, pH change and lactic acid production were monitored for the five isolates and it was found that acid production (titratable acidity) increased with time while pH reduced with time as is characteristic of LAB. LAB ferments the milk carbohydrate (lactose) to produce lactic acid that lead to decrease in pH and increases the value of titratable acidity. The obtained results revealed that isolates No. 9 performed as a better lactic acid producer as well as isolate No. 6, which are fungal strains (*Epicoccum* spp.) and isolate no. 28 (*Staphylococcus warneri*). Despite their acidifying activity, they were disqualified and isolates No. 24 and 27 (both *Lactobacillus plantarum* strains) were further evaluated for their acidifying activity. The obtained results revealed that the two *Lactobacillus plantarum* strains had a similar trend in acidification but neither could be characterized as fast, as they didn't reach a pH of 0.4 in 3 h at the optimum growth temperature[35]. These results were in agreement with those reported by Durlu-Ozkaya *et al.*[54], where it was found that *Lactobacillus* strains differ in their ability to reduce milk pH initially and there are some strains that don't change the pH of milk after 6 h of inoculation. *Lactobacillus plantarum* are known to produce lactic acid without inhibition and are facultative and heterofermentative microbes. Fermentation of sugars that cause pH decrease is important for clotting of milk. Besides, increasing acidity initiates desirable reactions and changes that leads to desired flavour, texture in fermented milk and also whey expulsion because there is a correlation between pH and whey expulsion from curd during cheese making process[55]. The two isolates were found to grow at high temperatures of 45°C. According to Ibourehama *et al.*[56], the capacity of bacteria to grow at high fermentation temperatures is a good characteristic, as it could be indicating an increased rate of growth and lactic acid production. Furthermore, the ability of these isolates to grow at high fermentation temperatures could decrease contamination by other microorganisms especially the pathogens. From the study by Yelnetty *et al.*[57], the proteolytic bacteria, *Lactobacillus plantarum* (similarly to what was obtained from this study) have the ability to ferment milk and could be used as starter culture to produce fermented milk both at household and commercial level.

Coagulation of the milk by the two *Lactobacillus plantarum* isolates (as starter cultures) was examined together with acid development. It was observed that coagulation of the milk by the two starter cultures started before 5 h and the milk became a firm coagulant in 18-24 h. At the early stages of fermentation, the pH value of each milk varied in the range of 6.47-6.64, which is typical of raw milk and decreased to 4.93-4.48 at the end of 24 h fermentation process. Seelee *et al.*[58] reported that pH value decreased during milk fermentation and the longer the fermentation time, the lower the pH value will be. The lowering of the pH to below 4.0 through acid production inhibits the growth of pathogenic microorganisms which can cause food spoilage, food poisoning and disease[59]. Therefore, the use of the isolated *Lactobacillus plantarum* strains as starter cultures will require fermentation of more than 24 h to further reduce the pH. By doing this, the shelf life of the fermented milk will be prolonged. However, *Lactobacillus plantarum* is a microorganism used mainly as a probiotic[60]. There are interesting data on anticarcinogenic effect of fermented foods showing potential role of *Lactobacillus plantarum* in reducing or eliminating procarcinogens and carcinogens in the alimentary canal[38,61], production of bacteriocins, antibacterial and antifungal properties, antioxidant, antimutagenic and health promoting properties[38]. Therefore, the isolated strains could be used as a starter culture to produce their own products, or they can be incorporated in other fermented products to confer the probiotic properties.

CONCLUSION

The results obtained in this study revealed the presence of *Lactobacillus plantarum* in *amabere amaruranu* where it could be playing a major role in the fermentation of *amabere amaruranu*. The technological characteristics of *Lactobacillus plantarum* as a potential starter culture was demonstrated and the organism can be used for product development as a starter culture/probiotic, however with further characterizations. In the milk product, there were members of *Epicoccum* spp. and *Staphylococcus warneri* whose benefits or adverse effects needs further studies.

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