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This is an author's accepted manuscript of an article published in the Brazilian Journal of Microbiology, DOI: 10.1007/s42770-021-00461-y, 2021.

The final publication is available at Springer via:

https://doi.org/10.1007/s42770-021-00461-y

Identification and characterisation of the lactic acid bacteria associated with the traditional fermentation of a dairy fermented product

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Abstract

The aim of this research was to identify the key lactic acid bacteria associated with the fermentation of dairy traditional fermented products for developing starter cultures for controlled fermentation. A total of 100 lactic acid bacteria (LAB) were isolated from dairy traditional fermented products. Samples were obtained from eight producers in the South East of Nigeria. Isolates were identified by phenotypic and genotypic techniques including rep-PCR genotyping and sequencing of the 16S rRNA, pheS and rpoA genes. Isolates were characterised for antimicrobial activity against foodborne pathogens, exopolysaccharide (EPS) production and survival at low pH and in the presence of bile salts. All isolates clustered into 11 distinct rep-PCR groups and were identified as *Lactobacillus fermentum* (40%), *Lactobacillus delbrueckii* (23%), *Streptococcus thermophilus* (22%), *Streptococcus infantarius* (10%), *Lactobacillus senioris* (2%), *Leuconostoc pseudomesenteriodes* (2%) and *Enterococcus thailandicus* (1%). *Lactobacillus fermentum* showed a broad spectrum antimicrobial activity and survival at low pH, while *Lactobacillus delbrueckii* was able to tolerate low pH and produce EPS. All isolates survived in vitro exposure to 1% (w/v) bile salts over a 3-h period. *L. fermentum*, *L. delbrueckii* and *S. thermophilus* could be used to simulate the fermentation of dairy traditional fermented products.

Keywords: Dairy traditional fermented product . Traditional milk products . Lactic acid bacteria . Potential starter cultures . Phenotypic and genotypic identification. Traditionally fermented foods

1. Introduction

Traditional fermented dairy products have been consumed for thousands of years and remain popular globally and across the African continent. In areas with limited access to electricity and cold storage facilities, fermentation is an important processing technique to extend the shelf life of milk, a highly perishable food. Fermentation also contributes to improving the organoleptic and nutritional characteristics of the final fermented product [1, 2, 3].

Nono is a naturally fermented, yoghurt-like product popular amongst many cattle owning and rearing pastoral communities in West Africa. The dairy traditional fermented product is made from cow's milk and is drunk as a refreshing nutritional drink or served as an accompaniment to *fura*, millet-based dough. Like other traditional fermented foods, dairy traditional fermented products play a significant role in the diet. It is a relatively cheap source of nutrients and an important source of income, particularly among women [4, 5]. It is also of cultural significance as traditional fermentation techniques are passed down generations, using modifications to obtain desired organoleptic characteristics.

Traditional processing of cow's milk for dairy traditional fermented product production varies across West Africa. It has been previously reported that dairy traditional fermented product is produced by fermenting raw milk fermented for *ca.* 24 h [6, 7] without any heat treatment. However, during this field study, it was observed that cow's milk is heat-treated among the Fulani who reside in the South-Eastern region of Nigeria, though not pasteurised, before fermentation.

Despite this important difference in fermentation practice, dairy traditional fermented production has similar characteristics to other naturally fermented African milk products such as *Amasi*, *Rob, Amabere Amaruranu* [8, 9, 10] concerning; use of backslopping, non-utilisation of starter cultures as well as small scale, household production. Reliance on spontaneous fermentation of milk leads to variability in the microbial consortium present in the milk and, subsequently, the final fermented product's quality. Poor hygiene during processing and handling can contribute to the contamination of the final

product [11]. Historically, dairy traditional fermented products have low acceptability outside pastoral communities due to their short shelf life and perceived low hygienic quality. It has been suggested that processing modifications such as pasteurised milk and controlled fermentation with well characterised Lactic acid bacteria can support improving marketability to a broader range of urban consumers [7].

Recently, there has been an increased focus on studying the microbiological and physicochemical properties of African fermented milk products. However, research on Nigerian dairy traditional fermented product has mainly concentrated on using phenotypic methods to assess microbiological quality [12, 13, 14] and less on the detailed identification of microflora associated with the fermentation. Information about fermentation temperature, time and pH change during dairy traditional fermented product production is also limited. An essential first step towards improving and standardising the fermentation process for dairy traditional fermented product*s is developing* functional starter cultures. An accurate understanding of the lactic acid bacteria involved during the fermentation is required to achieve this objective.

The purpose of this study was to enumerate, isolate and identify the predominant lactic acid bacteria (LAB) involved in the fermentation of cow milk for dairy traditional fermented product production using a combination of phenotypic and genotypic methods. Potential technological properties, including tolerance to acidic pH and bile salt, exopolysaccharides production and antimicrobial activity of LAB isolates against common foodborne pathogens, were also investigated.

2. Material and methods

2.1 Sampling

Samples of *nono* were collected in different areas of Abia State located in the South Eastern region of Nigeria, West Africa. A total of eight samples were collected from eight different producers. Two of these were from producers at Eket Islamic Mosque, Umuahia and six samples were collected from a farm settlement at Lokpa-Nta Fulani village. All samples were collected in sterile containers and kept

on ice before microbiological analysis. The pH of the samples was measured with a calibrated pH meter (Whatman PHA 2000, Portugal).

2.2 Microbiological analysis

2.2.1 Enumeration and isolation of presumptive lactic acid bacteria (LAB)

Enumeration and isolation of LAB from the dairy traditional fermented product were carried out using three different media, including deMan, Rogosa and Sharpe (MRS) agar (Oxoid, CM0361 Basingstoke, UK), MRS + 0.5 % L-Cysteine (MRSL) (C1276, Sigma-Aldrich, UK) and M17 Agar (Oxoid, CM0785). Plates were incubated anaerobically in an anaerobic jar (Oxoid, AG0025) with a gas kit (Oxoid, BR0038) added to create an anaerobic condition. Both MRSL and MRS agar plates were incubated at 37°C for 48 h, while M17 plates were incubated at 45°C for 48 h. After incubation, morphological characteristics of the colonies recovered from each agar were examined, and representative colonies were selected from appropriate dilutions. Bacteria were separately isolated and purified by streaking several times on the same media as appropriate. A single pure colony was picked aseptically and stored in a Microbank cryovial (Pro-Lab Diagnostics, Birkenhead, UK) at -20°C until required for further analysis.

2.2.2 Phenotypic characterisation of the isolated LAB

After growth on appropriate media, colony morphological characteristics such as size, shape and colour were examined. Cell morphology was examined by microscopy using a phase-contrast microscope (0.90 Dry Japan Nikon Eclipse E400). Bacteria were tested for Gram reaction using KOH (3 % w/v) as described by [15] and [16]. Isolates were also screened for the catalase enzyme reaction using 3 % (v/v) hydrogen peroxide (H3410, Sigma) and for the oxidase reaction using an oxidase reagent (Biomerieux®, 55635), on a strip of filter paper (Whatman No. 4, Whatman Plc., Kent, UK).

2.2.3 Genotypic characterisation of the isolates

a. Characterisation of the isolate by rep-PCR

A pure colony of each isolate was sub-cultured on tryptone soya agar (TSA, Oxoid, CM0131) and incubated for 24 h anaerobically at 37°C. Bacterial DNA was extracted using the Instagene matrix (Bio-Red 732-6030, Hercules, CA, USA) according to the manufacturer's instructions. Repetitive sequenced based PCR (rep-PCR) using the GTG5 (5'-GTG GTG GTG GTG GTG-3'; 5 pmol ml^{1}) primer as described by [17] was used to characterise isolates at subspecies level. For the amplification, the following programme was applied: 4 min at 94°C for initial denaturation, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 1 min and elongation at 65°C for 8 min. The amplification ended with a final extension at 65°C for 16 min.

The DNA fragments were separated by electrophoresis on 1.5 % (w/v) agarose gel (Bio-Rad, Hemel Hempstead UK) for 2 h in 1x Tris Borate-EDTA buffer (TBE, Sigma, UK) at 130 V. Gels were stained with ethidium bromide and DNA profiles visualised and recorded using a UV transilluminator gel documentation system (M-26X, UVP, Cambridge UK). Fingerprint patterns were analysed and clustered using the Bionumerics system (Dice's Coefficient of similarity, UPGMA; Applied Maths, Saint-Martens-Latem, Belgium).

b. Identification of bacteria by 16S rRNA, *phe*S and *rpo*A gene sequencing

To identify bacteria, the method described by [17] was used to sequence the 16S rRNA gene using primers pA (5' AGAGTTTGATCCTGGCTCAG-3') (100 mmol L^{-1}) and pE (5'-CCGTCAATTCCTTTGAGTTT-3'). The amplification was carried out under the following conditions: 5 min at 95°C for initial denaturation followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. A final extension was performed for 5 min at 72°C PCR products were purified using QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany 28104) following the manufacturer's instructions. A sequencing PCR using primer pD (5'-GTATTACCGCGGCTGCTG-3') was carried out under the following conditions: 95°C for 2 min, then 35 cycles at 96°C for 15 s, 40°C for 1 s and 60°C for 4 min before running on a gel (Source Bioscience, Cambridge, UK).

Where closely related species could not be differentiated by 16S rRNA gene sequencing, further identification was carried out by sequencing the *pheS* and *rpoA* genes according to the method described by [18]. Primers pheS-21-F (5'-CAY-CCNGCH-CGY-GAY-ATG-C-3') and pheS-23-R (5'-GGRTGR-ACC-ATV-CCN-GCH-CC-3') were used to direct the amplification of the *pheS* gene and *rpoA*-21-F (5'-CAY-CCNGCH-CGY-GAY-ATG-C-3') and *rpoA*-23-R (5'-GGRTGR-ACC-ATV-CCN-GCH-CC-3') the *rpoA* gene. The amplification programme consisted of (i) 5 min at 95°C, (ii) 3 cycles of 1 min at 95°C + 2 min 15 s at 46°C + 1 min 15 s at 72°C, (iii) 30 cycles of 35 s at 95°C + 1 min 15 s at 46°C + 1 min 15 s at 72°C and (iv) a final 7 min at 72°C. Isolates were identified to genus and species level by comparing sequences with those in the GenBank sequence database (NCBI, MD, USA). All 16S rRNA gene sequences were analysed using the EzBiocloud database [19].

2.3 Investigation of LAB technological properties

The isolates investigated included representative isolates of each rep-PCR group (Table 1). This included *Enterococcus thailandicus*, *Streptococcus infantarius, Lactobacillus senioris, Lactobacillus fermentum, Lactobacillus delbrueckii* subsp *indicus, Leuconostoc pseudomesenteroides* and *Streptococcus thermophilus*.

2.3.1 Screening of LAB for tolerance to acidic conditions

Isolates were sub-cultured on MRS or M17 agar, and a single pure colony was suspended in 1 ml of sterile Maximum Recovery Diluent (MRD) (Oxoid, Basingstoke, UK). The suspension was used to prepare an inoculum (with a final cell concentration of 10^7-10^8 CFU/ml (equivalent to 0.5 McFarland standard) using a Sensitre™ nephelometer (TREK Diagnostic Systems, West Sussex, UK). An acid resistance test was performed according to the method of [20]. One ml of each microbial suspension was inoculated into 9 ml of phosphate buffer solution (PBS) and adjusted to pH 3 and 7 using 2 M HCl and 2 M NaOH, respectively. Cultures were incubated at 37 °C for 3 h under anaerobic conditions. Cell viability was assessed every 30 min using a plate counting method on MRS and M17 agars. Plates were incubated anaerobically for 48 h at 37 °C, and viable cell counts were expressed as $\log_{10}$ CFU/ml.

160 **2.3.2 Screening of the LAB for tolerance to different % bile salt concentration.**

161 Bile salt tolerance of the isolates was ascertained in sterile PBS containing either no or 1.0 % (w/v) bile
162 salts, according to [20]. Inoculum preparation, medium inoculation, sampling and viable counts were
163 carried out as described above.

164 **2.3.3 Screening of LAB for exopolysaccharide (EPS) production**

165 This experiment was performed according to [21]. Skimmed milk agar plates containing 10 % (w/v)
166 skimmed milk, 1 % (w/v) sucrose (10020440, Fisher Scientific, UK), 0.5 % (w/v) yeast extract
167 (10225203, Fisher Scientific, UK), 1.5% (w/v) agar and 0.08 g/L ruthenium red (11103-72-3, Fisher
168 Scientific, UK) were prepared. Both LAB cultures from 48 h incubation and the control (*Enterococcus*
169 *casseliflavus*, Microbiology research Unit, London Metropolitan University) were streaked out on
170 separate plates, which were incubated anaerobically at 37°C for 48 h. Isolates unable to produce EPS
171 than the control appear as non-ropy, pink coloured colonies, while EPS producers exhibit a ropy,
172 whitish appearance [21].

173 **2.3.4 Screening LAB for antimicrobial activities against pathogenic bacteria**

174 **a. Inhibition of indicator of pathogenic bacteria using the spot test**

175 The spot test described by [18] was first used to evaluate the antimicrobial activity of the LAB isolates.
176 The activity of LAB isolates was tested against five indicator bacteria obtained from the culture
177 collection of the Microbiology Research Unit, School of Human Sciences, London Metropolitan
178 University (London, UK). These include *Samonella enteritidis serovar Typhimurium* variant DT124,
179 *Escherichia coli* NCTC 12900, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* CMCC 1930
180 and *Bacillus cereus* LMG 1356. An inoculum (2 µl) of each isolate was spotted on the surface of an MRS
181 agar plate and allowed to dry at room temperature for 30 min. All cultures were incubated
182 anaerobically at 37°C for 24 h. After the incubation time, 100 µl of each stock solution of an indicator
183 organism was inoculated into 10 ml TSB + 0.8% (w/v) agar and overlaid on the grown spotted cultures
184 of the LAB isolates. The overlaid plates were left to dry for 1 h at ambient temperature. Control plates

were set up by pouring the soft agar + indicator overlay on MRS agars without any test isolates spots. All plates were incubated aerobically for 24 - 48 h at 37°C, which is the optimum growth condition for the indicator bacteria. The diameter of the zone of inhibition was measured and recorded in mm.

b. Inhibition potential of cell-free supernatants (CFS) of LAB cultures against indicator bacteria using a spectrophotometric method

Antimicrobial activity resulting from a direct antagonism between the CFS of LAB isolates and indicator bacteria in liquid media was tested using the method described by [22] with some modifications. The CFS of LAB isolates was added 10% (v/v) to an inoculum of indicator bacteria in TSB. In the negative control, LAB CFS was substituted with 2 ml of MRS broth. The inhibitory activity of the CFS of the LAB isolates was determined by separately transferring (2 ml) of CFS of each test isolate into a universal bottle containing a mixture of 2 ml of each indicator bacterium culture and 16 ml of TSB. In the negative control, CFS was substituted with 2 ml of MRS broth. Cultures were then incubated aerobically for 24 h at 37°C. The optical density (OD) was measured at 540 nm (JENWAY 7315, Staffordshire, UK) by comparing the OD of the mixtures containing the indicator bacteria and the control mixture. However, before measuring the OD, the spectrophotometer was zeroed using a mixture of 2.6 ml TSB and 0.4 ml MRSB. Furthermore, to eliminate acid production as the sole antimicrobial property, an acid neutralisation test was conducted. The CFS of LAB isolates were prepared as previously described and neutralised with filtered sterilised 0.1M NaOH (Sigma, S8045) to increase the pH to 6.95 ± 0.1. The inhibitory effect of the neutralised CFS on the indicator bacteria was investigated using the spectrophotometric method as described above.

c. Screening potential of LAB isolates for production of antimicrobial peptides against indicator bacteria

Further characterisation of antimicrobial activity examined the possibility that LAB isolates investigated could produce antimicrobial peptides with broad-spectrum activity against the indicator bacteria. Each neutralised CFS was separately treated with Proteinase K (P2308, Sigma) to a final

concentration of 1 mg/ml. The treated CFS was incubated at 37°C for 2 h according to manufacturers' instructions. A negative control was set up using non-treated neutralised CFS for comparison. Inhibitory activities were determined using the spectrophotometric method as described above.

2.4 Data analysis

Each experiment was conducted at least two times, and data were analysed using Microsoft excel to determine the mean and standard deviation of the number of viable colonies. Also, statistically significant differences were set at $p \leq 0.05$ to compare the means using 1-way ANOVA. The results were expressed as mean ± standard deviation in $\log_{10}$ CFU/ml.

3. Results

3.1 Bacteria identification

After a 48 h fermentation period, all samples yielded bacterial counts of 10^7 CFU/ml. This corresponded with a decrease in pH from about 6.8 to 4.3 ± 0.23. A total of 100 bacteria were isolated from the eight samples of dairy traditional fermented product investigated. All isolates exhibited the primary features of LAB, i.e. Gram-positive, catalase-negative and oxidase negative. Microscopic observations revealed that a majority of the cells were rods arranged as chains, single and diplobacilli. Cocci present were arranged in chains, single, and diplococci, while some were V-shaped and coccobacillus.

All isolates were selected for molecular identification based on their phenotypic characteristics. Rep-PCR allowed differentiation of the isolates at interspecies and intraspecies levels into 11 different groups (Figure 1). The relatedness of the different group is variable (30-80 %), as shown in Figure 1. The combination of the 16S rRNA, pheS and rpoA gene sequencing allowed the identification of four genera of LAB, including *Lactobacillus*, *Streptococcus, Leuconostoc*, and *Enterococcus* and seven species including *Lactobacillus fermentum* (40%), *Lactobacillus senioris* (2%), *Lactobacillus delbrueckii* (23%), *Streptococcus thermophilus* (22%) *Streptococcus infantarius* (10%), *Leuconostoc pseudomesenteriodes* (2 %) and *Enterococcus thailandicus* (1%) (Table 1). Nucleotide sequence data

reported are available in the GenBank database under the **accession number MT956953 to MT956959**. *Lactobacillus fermentum* and *Lactobacillus delbruckii* were observed in all samples irrespective of the location and production site and were the most diverse species with 4 and 2 rep groups, respectively. Concerning production sites within the same location, it was observed that from Lokpa, in addition to the two common species, *Streptococcus thermophillus* was recovered from LO1, LO2, LO5 and LO6, *Streptococcus infantarius* from LO4 and LO5 and *Leuconostoc pseudomesenteroides* and *Lactobacillus senioris* from LO4 only. In Eket, additional species recovered were *Streptococcus infantarius* from E01, whereas *Enterococcus thailandicus*, *Lactobacillus senioris* and *Streptococcus thermophillus* were noticed in the sample from E02. The main difference between the two locations was the presence of *Leuconostoc pseudomesenteroides* in LO4 and *Enterococcus thailandicus* in EO1 (Table 1).

Using 16S rRNA gene sequencing, some bacteria could not be discriminated from closely related species. This was the case for isolates identified as *Enterococcus thailandicus* which could not be differentiated from *Enterococcus seguinicola*; *Leuconostoc pseudomesenteriodes* could not be differentiated from *Leuconostoc mesenteriodes*, and *Streptococcus infantarius*, which was not differentiable from *Streptococcus lutetiensis*. Such bacteria were all identified by rpoA and pheS genes sequencing (Table 1). All bacteria were identified with a percentage similarity of 98-100%.

3.2 Technological properties of LAB from dairy traditional fermented product

The effect of pH on the viability of the test isolates indicated that their tolerance to acid pH varied according to the isolate screened (Table 2). At pH 3, there was no different variation in viable cell count (about 10^7 CFU/ml) of *Lactobacillus fermentum* over the 3 hr test period while the viability of other isolates varied within the same test time (Table 2) compared to their numbers in the control at pH 7. *Streptococcus infantarius*, *Leuconostoc pseudomesenteroides* and *Streptococcus thermophilus* cultures lost their viability after 3 h of incubation while the viability of *Enterococcus thailandicus*, *Lactobacillus senioris* and *Lactobacillus delbrueckii* subsp. *indicus* were reduced respectively to 10^2

CFU/ml, 10^5 CFU/ml and 10^4 CFU/ml. Generally, *Lactobacillus fermentum* exhibited the highest viability count (10^7 CFU/ml) after 3 h incubation, while the least viability count (10^4 CFU/ml) was displayed by *Streptococcus thermophilus*. Except for *Streptococcus thermophilus*, all isolates showed good tolerance to bile, with no significant decrease in viable counts over the 3 h test period. Exposure to bile salts led to a 3 log decrease in *S. thermophilus* (Table 2).

Exopolysaccharides production also varied according to species. Some LAB isolates exhibited long, ropy strands, while others exhibited less ropy strand formation. Among the seven isolates screened, *Enterococcus thailandicus* and *Lactobacillus delbrueckii* subsp. *indicus* exhibited characteristics ascribed to EPS production by indicating the ropy whitish colonies similar to that of the control (Table 3). Other isolates showed no indication of EPS production except *Streptococcus thermophilus,* which showed less whitish colonies.

Lactic acid bacteria isolated from *nono* exhibited varying levels of inhibition against common Gram-positive and Gram-negative foodborne pathogens. It was observed that *Streptococcus thermophilus* did not inhibit any of the indicators screened. On the other hand, *Lactobacillus fermentum* exhibited, in general, a broad spectrum of inhibition against both Gram-positive and Gram-negative indicator bacteria (Table 3) with inhibition zones between 11 and 40 mm according to the indicator screened. Taking specific indicators into account, *Lactobacillus fermentum* exhibited the most potent inhibitory effect (21 - 30 mm inhibition zone) against *Salmonella enteritidis* while *Streptococcus infantarius Lactobacillus senioris* exhibited the most substantial inhibitory effect (21 - 30 mm inhibition zone) against *Escherichia coli*. All LAB except *Streptococcus thermophilus* showed the same degree of inhibition (11-20 mm inhibition zone) against *Staphylococcus aureus* and *Listeria monocytogenes*. Also, *Bacillus cereus* was the most sensitive indicator with the largest clear inhibition zones on average of (21 mm – 40 mm, Table 3).

All LAB showed varying degrees of antimicrobial activity due to direct antagonism between the CFS and indicator bacteria in liquid media. The pH of the CFS dropped from 6.0 (MRS broth) and 6.8 (M17

broth) to 3.97, 4.14, 4.16, 4.26, 4.29, 4.29 and 6.07 for the CFS of broth cultures of *Lactobacillus delbrueckii* subsp *indicus, Lactobacillus fermentum*, *Streptococcus infantarius*, *Lactobacillus senioris*, *Enterococcus thailandicus*, *Leuconostoc pseudomesenteroides*, and *Streptococcus thermophilus* respectively. The CFS of *Lactobacillus fermentum* exhibited the highest antimicrobial effect against all indicators screened, followed by *Lactobacillus senioris* (Figures 2). Furthermore, *Streptococcus thermophilus* exhibited the least effect on the growth of the indicator bacteria, particularly against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*.

Overall, inhibitory activities observed from the CFS of test isolates were removed after neutralisation. For example, neutralised CFS of *Lactobacillus senioris* lost its effect on the growth of all the indicator bacteria screened (Figure 2). When their CFS were neutralised, other test isolates retained their inhibition effect only against *Bacillus cereus* compared to non – neutralised CFS (Figure 2b). The antimicrobial effect of *Streptococcus thermophilus* against *Salmonella enteritidis* was also not observed to be influenced by neutralisation (Figure 2c).

Further characterisation to determine the potential of isolates to produce antimicrobial peptides against the indicators screened showed that the inhibitory activities observed from the neutralised CFS against *Bacillus cereus* were lost after proteolytic enzyme (proteinase K) treatment. All treated CFS exhibited antimicrobial effect against *Staphylococcus aureus* (Figure 2d).

Generally, *Streptococcus infantarius* exhibited the most potent antimicrobial activity against *Listeria monocytogenes* compared to other test isolates. *Lactobacillus senioris* and *Streptococcus thermophilus* did not show inhibition potential against *Listeria monocytogenes*.

Discussion

The isolation and identification of LAB from the dairy traditional fermented product, a traditional fermented milk product, was evaluated. Microbial counts of LAB in *nono* ranged between 1.34×10^7 and 8.76×10^7 and are similar to those reported for other African fermented milk products with counts of 10^6 - 10^8 [6, 23, 9]. The reduction in pH observed in fermented milk products like *nono* is associated

with the production of lactic acid and other types of organic acids by fermenting lactic acid bacteria. These observations are similar to other studies on traditional African fermented milks, which have been reported to range from 3.2 – 4.8 [1]. A study on *kule naoto*, a Maasai traditional fermented milk from Kenya, reported a final pH between 4.17-5.16 [24]. In *nunu*, fermented milk from Ghana, a much lower pH value of 3.1 was reported [6]. These differences may be related to the consortium of lactic acid bacteria involved in the fermentation and their particular technological properties such as acid production and fermentation time [25].

Rep-PCR was adequate for the differentiation of LAB isolates at interspecies and intraspecies levels and enabled the diversity of the lactic acid bacteria responsible for the fermentation to be explored. A combination of 16S rRNA and other housekeeping genes is necessary to provide accurate bacterial identity and has been demonstrated in identifying LAB from other fermented food materials [18, 26]. The current study demonstrated that different genera, species, and subspecies of LAB, including *Lactobacillus fermentum*, *Lactobacillus senioris*, *Lactobacillus delbrueckii* subsp *indicus*, *Streptococcus thermophilus*, *Streptococcus infantarius*, *Leuconostoc pseudomesenteriodes* and *Enterococcus thailandicus* are involved in the fermentation of cow milk for *nono* production. Results also indicated that location might influence the microbial profile as *Leuconostoc pseudomesenteroides* and *Enterococcus thailandicus* were observed only in Lokpa and Eket, respectively.

The predominance of *Lactobacillus fermentum* in traditional African fermented milk products is in agreement with other reports [27, 6, 24]. Unlike other authors who have noted *Lactobacillus plantarum* as a dominant LAB species in African traditional fermented cow milk products, [6, 28, 9, 29] this was not our observation. *Lactobacillus plantarum* is usually associated with the fermentation of vegetables and root crops [30], and it has been suggested that its presence in milk may be due to contamination [1]. This observed difference could be attributed, at least in part, to the variation in methods used to isolate and identify LAB from fermented milk products. For example, MRS agar was shown to be a suitable medium for the enumeration and recovery of *Lactobacillus* spp. [31, 4, 32, 33, 34, 35] while M17 agar is more selective for *Streptococcus* species such as *Streptococcus thermophilus*

and lactococci [36, 37]. Also, many of these studies rely on phenotyping alone for identification which can be unreliable in providing accurate identification of bacterial species. To our knowledge, this is the first study using molecular techniques to characterise the microorganisms involved in the fermentation of *nono* consumed in Nigeria.

Generally, during the production and consumption of fermented milk, bacteria involved are exposed to different environmental conditions such as acids, bile, oxygen and oxygen-derived radicals; heat and cold stress; which could negatively affect their viability and functionality [38]. In this study, *Lactobacillus fermentum* followed by *Lactobacillus senioris* showed good viability at pH 3 for 3 h incubation than other tested strains supporting the results of the study of [39], which demonstrated that *Lactobacillus* species are more tolerant to the acid environment than the other genera of LAB. Hence, this property makes *Lactobacillus* species abundant in the final phases of many food fermentations.

Tolerance to bile is considered one of the essential properties required for probiotic bacteria to survive in the small intestine [40]. In this study, all the tested strains showed good tolerance to bile. Other similar studies have assessed this at different concentrations from 0.5 % (w/v) up to 2% (w/v). For instance, Giri [41] observed the higher tolerance of LAB isolated from fish intestine at 2% bile concentrations. Maragkoudakis [42] explained that *Lactobacillus* strains of dairy origin survived exposure to 0.3 % w/v bile salts for 4 h when screening their probiotic potential.

Extracellular polysaccharides forming strains have some advantages of improving texture, avoiding syneresis and increasing the viscosity of the yoghurt. In addition, EPS-forming LAB have been used to improve the rheological characteristics of dairy products. In this study, *Enterococcus thailandicus* and *Lactobacillus delbrueckii* subsp *indicus* showed higher EPS production while *Streptococcus thermophilus* showed less EPS production. The current observation is similar to the observations from Patil *et al*. [43]. The authors observed that EPS production from dairy isolates varies among species. Also, the presence of additional metabolites in milk can influence EPS production. For instance, the addition of glucose or sucrose to milk and milk ultrafiltrate increased EPS production by ropy strains

of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. casei* subsp. *casei*. [44] also observed enhanced growth and EPS production by *Strep. thermophilus* strains in enriched milk medium supplemented with 1.0% peptone and 0.5% yeast extract, and these observations are similar to observations in this study concerning the medium used for EPS production.

In Africa, traditional fermented products such as dairy traditional fermented products remain a cottage level industry. Due to limited training, awareness and practice of Hazard Analysis and Critical Control Point (HACCP) and Good Manufacturing Practice (GMP) by producers and food handlers, the presence of pathogenic bacteria cannot be ruled out [45]. Recent reports indicate that traditional fermented products available for retail sale can serve as vehicles for pathogenic bacteria [1, 46]; therefore, antimicrobial activity is an important technological aspect when selecting LAB starter cultures for the controlled production of fermented dairy products.

Lactic acid bacteria from *nono* were characterised based on their antimicrobial properties against three Gram-positive (*Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*) and two Gram-negative (*Salmonella enteritidis, Escherichia coli*) indicators of foodborne pathogens. The study also aimed to accurately attribute antimicrobial properties due to one or a combination of competition for nutrients, acid production and production of antimicrobial peptides. The spot test results showed that six out of seven LAB isolates exhibited varying levels of inhibition against common Gram-positive and Gram-negative foodborne pathogens. Notably, *Lactobacillus fermentum* exhibited a broad spectrum of inhibition against both types of indicator bacteria. The ability of *Lactobacillus fermentum* strains isolated from fermented milk products to show broad-spectrum inhibitory activity has been reported by other authors [47, 48]. *Bacillus cereus* was the most sensitive indicator when tested against all LAB isolates that showed antimicrobial activity in the spot test. This strong antagonistic activity of LAB isolates from fermented milk products against strains of *Bacillus cereus* has been reported by other authors [49, 50, 51]. This result is promising as any potential starter needs must be able to inhibit the growth of spore-forming bacteria, thereby improving the safety and quality of the product.

In this study, a more sensitive assay based on a spectrophotometric method showed that most CFS of the LAB isolates from dairy traditional fermented product inhibited the growth of the indicator bacteria in broth cultures. The growth of *Listeria monocytogenes* was notably impeded in the presence of CFS. This is of particular interest as starter cultures for fermented milk products that show antilisterial activity are important in the food and dairy industries. *Listeria* spp. is commonly associated with dairy products [49] with related safety issues. The ability of the CFS to inhibit the growth of the indicators shows that the antimicrobial effect cannot be solely attributed to competition for nutrients. Thus, the exact mechanism of inhibitory activities was further evaluated to ascertain if inhibition was due to factors such as the production of acid or antimicrobial proteins. In general, the bacteriostatic effect of the test isolates on the indicator organisms was removed after neutralisation of the CFS, indicating that acid production was most likely the main antimicrobial effect. This observation has been reported in other studies [52, 53]. [54] reported that none of the neutralised CFS from LAB strains studied showed antimicrobial activity against any of the Gram-negative pathogens tested. This is the case in the current study, as observed in *Streptococcus infantarius*, *Lactobacillus senioris* and *Lactobacillus fermentum* against *Bacillus cereus*.

The removal or reduction of inhibition after treatment with proteolytic enzymes in many cases suggests that some of the antimicrobial activities observed are likely due to the action of antimicrobial peptides such as bacteriocins or bacteriocin like inhibitory substances (BLIS) [55, 56, 23]. Although in this study, the addition of proteolytic enzymes to the neutralised CFS was associated with a decrease in the inhibitory effect of LAB isolates, both *Lactobacillus senioris* and *Leuconostoc pseudomesenteroides* maintained inhibitory activities against *E. coli* after neutralisation.

Conclusion

Naturally fermented milk products like nono are produced by spontaneous fermentation with related issues of inconsistency in quality, safety, nutritional and organoleptic properties. The selection of multifunctional starter culture for the development of controlled fermentation could address these

problems and contribute to improved food security in Africa by increasing the availability of animal products and providing a source of income for producers. Potential lactic acid bacteria for use as multifunctional starter cultures for *nono* production include *L. fermentum, L*. *delbrueckii* and *S. thermophilus*. Further investigation should be carried out to develop appropriate conditions for up-grading this traditionally fermented milk product.

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575 **Table 1 Identity of the LAB isolated from a dairy traditional fermented product with accession number**

576 **SUB8090459/ MT956953-MT956959**

Isolate code	*Sample Location	Rep-PCR Group	Identification by 16S rDNA, *phe*S, *rpo*A gene sequencing
1	LO1	A	*Lactobacillus fermentum*
2	LO1	B	*Lactobacillus fermentum*
33	LO1	B	*Lactobacillus fermentum*
35	LO1	B	*Lactobacillus fermentum*
54	LO1	B	*Lactobacillus fermentum*
36	LO1	D	*Lactobacillus fermentum*
37	LO1	D	*Lactobacillus fermentum*
69	LO1	G	*Streptococcus thermophillus*
71	LO1	G	*Streptococcus thermophillus*
74	LO1	G	*Streptococcus thermophillus*
70	LO1	H	*Streptococcus thermophillus*
73	LO1	H	*Streptococcus thermophillus*
72	LO1	H	*Streptococcus thermophillus*
34	LO1	J	*Lactobacillus delbrueckii subsp. indicus*
53	LO1	J	*Lactobacillus delbrueckii subsp. indicus*
17	LO1	J	*Lactobacillus delbrueckii subsp. indicus*
5	LO2	A	*Lactobacillus fermentum*
19	LO2	A	*Lactobacillus fermentum*
3	LO2	B	*Lactobacillus fermentum*
38	LO2	B	*Lactobacillus fermentum*
55	LO2	B	*Lactobacillus fermentum*
56	LO2	D	*Lactobacillus fermentum*
57	LO2	D	*Lactobacillus fermentum*
75	LO2	G	*Streptococcus thermophillus*
76	LO2	H	*Streptococcus thermophillus*
4	LO2	J	*Lactobacillus delbrueckii subsp. indicus*
18	LO2	J	*Lactobacillus delbrueckii subsp. indicus*
39	LO2	J	*Lactobacillus delbrueckii subsp. indicus*
58	LO3	A	*Lactobacillus fermentum*
7	LO3	A	*Lactobacillus fermentum*
20	LO3	A	*Lactobacillus fermentum*
40	LO3	A	*Lactobacillus fermentum*
41	LO3	A	*Lactobacillus fermentum*
59	LO3	A	*Lactobacillus fermentum*
6	LO3	J	*Lactobacillus delbrueckii subsp. indicus*
8	LO3	J	*Lactobacillus delbrueckii subsp. indicus*
21	LO4	B	*Lactobacillus fermentum*
61	LO4	B	*Lactobacillus fermentum*
9	LO4	E	*Leuconostoc pseudomesenteroides*
42	LO4	E	*Leuconostoc pseudomesenteroides*
43	LO4	F	*Lactobacillus senioris*

577 *LO1, LO2, LO3, LO4, LO5 and LO6: Production sites from Lokpa

578 EO1 and EO2: Production sites from Eke.

579 **Table 1** *(contd)* Identity of the LAB isolated from a dairy traditional fermented product

Isolate code	*Sample Location	Rep-PCR Group	Identification by 16S rDNA, *phe*S, *rpo*A gene sequencing
79	LO4	I	*Streptococcus infantarius*
80	LO4	I	*Streptococcus infantarius*
77	LO4	I	*Streptococcus infantarius*
78	LO4	I	*Streptococcus infantarius*
10	LO4	I	*Streptococcus infantarius*
44	LO4	I	*Streptococcus infantarius*
22	LO4	J	*Lactobacillus delbrueckii subsp. indicus*
60	LO4	J	*Lactobacillus delbrueckii subsp. indicus*
46	LO5	A	*Lactobacillus fermentum*
47	LO5	A	*Lactobacillus fermentum*
12	LO5	A	*Lactobacillus fermentum*
82	LO5	G	*Streptococcus thermophillus*
88	LO5	G	*Streptococcus thermophillus*
86	LO5	H	*Streptococcus thermophillus*
81	LO5	H	*Streptococcus thermophillus*
87	LO5	H	*Streptococcus thermophillus*
83	LO5	I	*Streptococcus infantarius*
84	LO5	I	*Streptococcus infantarius*
85	LO5	I	*Streptococcus infantarius*
11	LO5	J	*Lactobacillus delbrueckii subsp. indicus*
23	LO5	J	*Lactobacillus delbrueckii subsp. indicus*
24	LO5	J	*Lactobacillus delbrueckii subsp. indicus*
45	LO5	J	*Lactobacillus delbrueckii subsp. indicus*
14	LO6	A	*Lactobacillus fermentum*
63	LO6	A	*Lactobacillus fermentum*
13	LO6	C	*Lactobacillus fermentum*
25	LO6	C	*Lactobacillus fermentum*
26	LO6	C	*Lactobacillus fermentum*
48	LO6	C	*Lactobacillus fermentum*
50	LO6	C	*Lactobacillus fermentum*
62	LO6	C	*Lactobacillus fermentum*
92	LO6	G	*Streptococcus thermophillus*
89	LO6	G	*Streptococcus thermophillus*
91	LO6	G	*Streptococcus thermophillus*
90	LO6	H	*Streptococcus thermophillus*
49	LO6	J	*Lactobacillus delbrueckii subsp. indicus*
28	EO1	A	*Lactobacillus fermentum*
29	EO1	A	*Lactobacillus fermentum*
51	EO1	A	*Lactobacillus fermentum*
64	EO1	A	*Lactobacillus fermentum*

580 *LO1, LO2, LO3, LO4, LO5 and LO6: Production sites from Lokpa

581 EO1 and EO2: Production sites from Eke

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583 **Table 1** (*contd*) Identity of the LAB isolated from the dairy traditional fermented product

Isolate code	*Sample Location	Rep-PCR Group	Identification by 16S rDNA, *phe*S, *rpo*A gene sequencing
95	EO1	I	*Streptococcus infantarius*
30	EO1	J	*Lactobacillus delbrueckii subsp. indicus*
65	EO1	J	*Lactobacillus delbrueckii subsp. indicus*
27	EO1	J	*Lactobacillus delbrueckii subsp. indicus*
94	EO1	J	*Lactobacillus delbrueckii subsp. indicus*
93	EO1	J	*Lactobacillus delbrueckii subsp. indicus*
15	EO1	J	*Lactobacillus delbrueckii subsp. indicus*
31	EO2	A	*Lactobacillus fermentum*
32	EO2	A	*Lactobacillus fermentum*
68	EO2	A	*Lactobacillus fermentum*
67	EO2	F	*Lactobacillus senioris*
97	EO2	G	*Streptococcus thermophillus*
96	EO2	G	*Streptococcus thermophillus*
99	EO2	G	*Streptococcus thermophillus*
100	EO2	G	*Streptococcus thermophillus*
98	EO2	G	*Streptococcus thermophillus*
16	EO2	J	*Lactobacillus delbrueckii subsp. indicus*
66	EO2	J	*Lactobacillus delbrueckii subsp. indicus*
52	EO2	K	*Enterococcus thailandicus*

584 *LO1, LO2, LO3, LO4, LO5 and LO6: Production sites from Lokpa

585 EO1 and EO2: Production sites from Eke

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Table 2 Survival of the LAB from a dairy traditional fermented product in low pH and their tolerance to bile salt

Isolates code	Species	Viable count (CFU/ml)							
		pH 7		pH 3		0 % bile salt		1 % bile salt	
		0 h	3 h	0 h	3 h	0 h	3 h	0 h	3 h
52	*Enterococcus thailandicus*	6.33 ± 0.08^{bc}	6.18 ± 0.20^{bc}	6.31 ± 0.04^{bc}	2.90± 0.26^{e}	7.68 ± 0.07^{ab}	7.75 ± 0.09^{ab}	7.55 ± 0.10^{ab}	7.61 ±0.11^{ab}
11	*Lactobacillus delbrueckii subsp. indicus*	6.68 ± 0.04^{b}	5.35 ± 0.06^{c}	5.87 ± 0.07^{bc}	4.26± 0.03^{d}	5.60 ± 0.07^{bc}	5.73 ± 0.14^{bc}	5.56 ± 0.14^{bc}	5.23 ± 0.09^{c}
13	*Lactobacillus fermentum*	7.85 ± 0.00^{ab}	7.48 ± 0.02^{ab}	7.47 ± 0.01^{ab}	7.32± 0.00^{b}	8.29 ± 0.05^{a}	8.29 ± 0.35^{a}	8.25 ± 0.10^{a}	7.18 ± 0.81^{b}
43	*Lactobacillus senioris*	7.78 ± 0.13^{ab}	7.68 ± 0.06^{ab}	7.62 ± 0.02^{ab}	5.98±0.19^{bc}	7.59 ± 0.12^{ab}	7.65 ± 0.05^{ab}	7.66 ± 0.03^{ab}	7.69 ± 0.02^{ab}
9	*Leuconostoc pseudomesenteroides*	6.56 ± 0.15^{b}	6.12 ± 0.02^{bc}	6.33 ± 0.16^{bc}	-	7.94 ± 0.14^{ab}	7.29 ± 0.16^{b}	7.96 ± 0.17^{ab}	6.39 ± 0.16^{bc}
10	*Streptococcus infantarius*	6.86 ± 0.04^{b}	6.46 ± 0.11^{b}	6.38 ± 0.21^{bc}	-	8.18 ± 0.47^{a}	7.84 ± 0.01^{ab}	7.70 ± 0.07^{ab}	7.47 ± 0.03^{ab}
73	*Streptococcus thermophilus*	5.94 ± 0.03^{bc}	4.51 ± 0.03^{c}	4.37 ± 0.18^{d}	-	6.32 ±0.06^{bc}	5.26 ±0.07^{c}	5.40 ± 0.08^{bc}	2.86 ± 0.10^{e}

Data represent the mean of the viable count in two experiments expressed as mean ± standard deviation in $\log_{10}$ Cfu/ml.

Data were considered significantly different (rows/columns) when $P < 0.05$.