

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

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20 **Abstract**

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

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

## 38 1. Introduction

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

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

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

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

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

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

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

## 82 **2. Material and methods**

### 83 **2.1 Sampling**

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

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

## 90 **2.2 Microbiological analysis**

### 91 **2.2.1 Enumeration and isolation of presumptive lactic acid bacteria (LAB)**

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

### 102 **2.2.2 Phenotypic characterisation of the isolated LAB**

103 After growth on appropriate media, colony morphological characteristics such as size, shape and  
104 colour were examined. Cell morphology was examined by microscopy using a phase-contrast  
105 microscope (0.90 Dry Japan Nikon Eclipse E400). Bacteria were tested for Gram reaction using KOH (3  
106 % w/v) as described by [15] and [16]. Isolates were also screened for the catalase enzyme reaction  
107 using 3 % (v/v) hydrogen peroxide (H3410, Sigma) and for the oxidase reaction using an oxidase  
108 reagent (Biomerieux®, 55635), on a strip of filter paper (Whatman No. 4, Whatman Plc., Kent, UK).

### 109 **2.2.3 Genotypic characterisation of the isolates**

#### 110 **a. Characterisation of the isolate by rep-PCR**

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

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

#### 125 **b. Identification of bacteria by 16S rRNA, *pheS* and *rpoA* gene sequencing**

126 To identify bacteria, the method described by [17] was used to sequence the 16S rRNA gene using  
127 primers pA (5' AGAGTTTGATCCTGGCTCAG-3') (100 mmol L<sup>-1</sup>) and pE (5'-CCGTCGAATTCCTTTGAGTTT-3').  
128 The amplification was carried out under the following conditions: 5 min at 95°C for initial denaturation  
129 followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1 min, and elongation at  
130 72°C for 1 min. A final extension was performed for 5 min at 72°C PCR products were purified using  
131 QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany 28104) following the manufacturer's  
132 instructions. A sequencing PCR using primer pD (5'-GTATTACCGCGGCTGCTG-3') was carried out under  
133 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  
134 min before running on a gel (Source Bioscience, Cambridge, UK).

135 Where closely related species could not be differentiated by 16S rRNA gene sequencing, further  
136 identification was carried out by sequencing the *pheS* and *rpoA* genes according to the method  
137 described by [18]. Primers pheS-21-F (5'-CAY-CCNGCH-CGY-GAY-ATG-C-3') and pheS-23-R (5'-  
138 GGRTGR-ACC-ATV-CCN-GCH-CC-3') were used to direct the amplification of the *pheS* gene and *rpoA*-  
139 21-F (5'-CAY-CCNGCH-CGY-GAY-ATG-C-3') and *rpoA*-23-R (5'-GGRTGR-ACC-ATV-CCN-GCH-CC-3') the  
140 *rpoA* gene. The amplification programme consisted of (i) 5 min at 95°C, (ii) 3 cycles of 1 min at 95°C +  
141 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  
142 at 72°C and (iv) a final 7 min at 72°C. Isolates were identified to genus and species level by comparing  
143 sequences with those in the GenBank sequence database (NCBI, MD, USA). All 16S rRNA gene  
144 sequences were analysed using the EzBiocloud database [19].

### 145 **2.3 Investigation of LAB technological properties**

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

#### 150 **2.3.1 Screening of LAB for tolerance to acidic conditions**

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



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

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

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

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

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

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

## 213 **2.4 Data analysis**

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

## 218 **3. Results**

### 219 **3.1 Bacteria identification**

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

227 All isolates were selected for molecular identification based on their phenotypic characteristics. Rep-  
228 PCR allowed differentiation of the isolates at interspecies and intraspecies levels into 11 different  
229 groups (Figure 1). The relatedness of the different group is variable (30-80 %), as shown in Figure 1.  
230 The combination of the 16S rRNA, pheS and rpoA gene sequencing allowed the identification of four  
231 genera of LAB, including *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Enterococcus* and seven  
232 species including *Lactobacillus fermentum* (40%), *Lactobacillus senioris* (2%), *Lactobacillus delbrueckii*  
233 (23%), *Streptococcus thermophilus* (22%) *Streptococcus infantarius* (10%), *Leuconostoc*  
234 *pseudomesenteriodes* (2 %) and *Enterococcus thailandicus* (1%) (Table 1). Nucleotide sequence data

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

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

### 252 **3.2 Technological properties of LAB from dairy traditional fermented product**

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

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

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

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

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

285 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*  
286 *delbrueckii* subsp *indicus*, *Lactobacillus fermentum*, *Streptococcus infantarius*, *Lactobacillus senioris*,  
287 *Enterococcus thailandicus*, *Leuconostoc pseudomesenteroides*, and *Streptococcus thermophilus*  
288 respectively. The CFS of *Lactobacillus fermentum* exhibited the highest antimicrobial effect against all  
289 indicators screened, followed by *Lactobacillus senioris* (Figures 2). Furthermore, *Streptococcus*  
290 *thermophilus* exhibited the least effect on the growth of the indicator bacteria, particularly against  
291 *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus*.

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

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

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

## 305 **Discussion**

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

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

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

327 The predominance of *Lactobacillus fermentum* in traditional African fermented milk products is in  
328 agreement with other reports [27, 6, 24]. Unlike other authors who have noted *Lactobacillus*  
329 *plantarum* as a dominant LAB species in African traditional fermented cow milk products, [6, 28, 9, 29]  
330 this was not our observation. *Lactobacillus plantarum* is usually associated with the fermentation of  
331 vegetables and root crops [30], and it has been suggested that its presence in milk may be due to  
332 contamination [1]. This observed difference could be attributed, at least in part, to the variation in  
333 methods used to isolate and identify LAB from fermented milk products. For example, MRS agar was  
334 shown to be a suitable medium for the enumeration and recovery of *Lactobacillus* spp. [31, 4, 32, 33,  
335 34, 35] while M17 agar is more selective for *Streptococcus* species such as *Streptococcus thermophilus*

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

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

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

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

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

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

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



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

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

## 408 **Conclusion**

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

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

## 417 **References**

- 418 1. Jans C, Meile L, Kaindi DWM, Kogi-Makau W, Lamuka P, Renault P, Kreikemeyer B, Lacroix  
419 C, Hattendorf J, Zinsstag J, Schelling E, Fokou G, Bonfoh B (2017) African fermented dairy  
420 products – Overview of predominant technologically important lactic acid bacteria focusing on  
421 African *Streptococcus infantarius* variants and potential future applications for enhanced food safety  
422 and security. Int J Food Microbiol 250:27–36.
- 423 2. Gadaga TH, Mutukumira AN, Naruhus JA (2000) Enumeration and identification of yeast isolated  
424 from Zimbabwean traditional fermented milk. Int Dairy J 10:459-466.
- 425 3. Narvhus JA, Gadaga TH (2003) The role of interaction between yeasts and lactic acid bacteria in  
426 African fermented milks: a review. Int J Food Microbiol 86:51-60.
- 427 4. Gonfa A, Foster HA, Holzapfel WH (2001) Field survey and literature review on traditional  
428 fermented milk product of Ethiopia. Int J Food Microbiol 68:173-186.
- 429 5. Oyewole O.B (1997) Lactic fermented food in Africa and their benefit. Food Control 8:289-297.
- 430 6. Akabanda F, Owusu-Kwarteng J, Glover ALK, Tano-Debrah K, Nelson DS, Jespersen L (2013)  
431 Taxonomic and molecular characterisation of lactic acid bacteria and yeast in Nunu, a Ghanaian  
432 fermented milk product. Food Microbiol 34:277-283.
- 433 7. Okonkwo OI (2011) Microbiological analysis and safety evaluation of dairy traditional fermented  
434 product: A fermented milk product consumed in most parts of Northern, Nigeria. Int J Dairy Science  
435 6:181-189.

- 436 8. Abdelqadir WS, Hamad SH, MØller LP, Jakobsen M (2001) Characterisation of the dominant  
437 microbiota of Sudanese fermented milk "Rob." Int Dairy J 11:63-70.
- 438 9. Nyambane B, Thari W, Wangoh J, Njage P (2014) Lactic acid bacteria and yeasts involved in the  
439 fermentation of *amabere amaruranu*, a Kenyan fermented milk. Food Sci Nutr 2:692-699.
- 440 10. Osvik RD, Sperstad S, Breines EM, Hareide E, Godfroid J, Zhou Z, Ren P, Geoghegran C, Holzapfel  
441 W, Einar R (2013) Bacterial diversity of *amasi*, a South African fermented milk product, determined  
442 by clone library and denaturing gradient gel electrophoresis analysis. African J Microbiol Res 7:4146-  
443 4168.
- 444 11. Okeke OFI, Okwori AEJ (2011) Occurrence of pathogenic *Yersinia* species in locally fermented  
445 cow milk (Dairy traditional fermented product) in Jos, Nigeria. Nigeria J Biotech 23:45-52.
- 446 12. Egwaikhide PA, Malu PS, Lawal U, Adelagun RO, Andrew C (2014) Physico-chemical and  
447 microbiological analysis of fermented cow milk (Dairy traditional fermented product) consumed  
448 within Kaduna town, North-Western Nigeria. Food Sci Qual Manag 29:1-44.
- 449 13. Obande GA, Azua ET (2013) Extent of microbial contamination of Dairy traditional fermented  
450 product product product, fresh cow milk and yoghurt sold in Makurdi Benue State, Nigeria. J  
451 Microbiol Biotechnol Res 3:6-14.
- 452 14. Yabaya A, Manga SS, Lucy M, Alhassan HM (2012) Bacteriology quality of fermented milk sold  
453 locally in Samaru and Sabongari market, Zaria Nigeria. Continental J Microbiol 6:14-18.
- 454 15. Lanyi B (1988) Classical and rapid identification methods for medically important bacteria. In  
455 Colwell RR, Grigorova R (ed) Methods in microbiology, Vol 19. Academic Press Inc, USA, pp 1-67.
- 456 16. Powers E M (1995) Efficacy of the Ryu non-staining KOH technique for rapidly determining gram  
457 reactions of food-borne and waterborne bacteria and yeasts. App Env Microbiol 61:3756-3758.

- 458 17. Ouoba L.I.I, Parkouda C, Diawara B, Scotti C, Varnam, AH (2008) Identification of *Bacillus spp.*  
459 from Bikalga fermented seed of Hibiscus sabdariffa: phenotypic and genotypic characterisation. J  
460 Applied Microbiol 104:122-131.
- 461 18. Anyogu A, Awamaria B, Sutherland JP, Ouoba LII (2014) Molecular characterisation and  
462 antimicrobial activity of bacteria associated with submerged lactic acid cassava fermentation. Food  
463 Control. 39:119-127.
- 464 19. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: A  
465 taxonomically united database of 16S rRNA and whole-genome assemblies. Int J Syst Evol  
466 Microbiol 67:1613-1617.
- 467 20. Prasad J, Gill H, Smart J, Gopal P (1998) Selection and characterisation of *Lactobacillus* and  
468 *Bifidobacterium* strains for use as probiotics. Int Dairy J. 8:993–1002.
- 469 21. Mora D, Fortina MG, Parini C, Ricci G, Gatti M, Giraffa G, Manachini PL (2002) Genetic diversity and  
470 technological properties of *Streptococcus thermophilus* strains isolated from dairy products. J Applied  
471 Microbiol 93:278-287.
- 472 22. Lash BW, Mysliwiec TH, Gourama H (2005) Detection and partial characterisation of a broad  
473 range bacteriocin produced by *Lactobacillus plantarum* (ATCC 8014). Food Microbiol 22:199-2014.
- 474 23. Mohamed SSD, Ijah UJJ (2013) Isolation and screening of lactic acid bacteria from fermented milk  
475 product for bacteriocin production. Ann Food Sci-Tech 4:122-124.
- 476 24. Mathara JM, Schillinger U, Kutima PM, Mbugua SK, Holzapfel WH (2004) Isolation, identification,  
477 and characterisation of the dominant Lactic acid bacteria Kule naoto: The Maasai traditional  
478 fermented milk in Kenya. Int J Food Microbiol 94:269-278.
- 479 25. Rezac S, Kok RC, Heermann M, Hutkins C (2018) Fermented food as a dietary source of live  
480 organisms. Front Microbiol 9:1-29.

- 481 26. Ahaotu NN, Anyogu A, Obioha P, Aririatu L, Ibekwe VI, Oranusi S, Sutherland JP, Ouoba LII (2017)  
482 Influence of soy fortification on microbial diversity during cassava fermentation and subsequent  
483 physicochemical characteristics of garri. *Food Microbiol.* 66:165 – 172.
- 484 27. Abdelgadir W, Nielsen DS, Hamad S, Jakobsen M (2008) A traditional Sudanese fermented  
485 camel's milk product, Gariss, as a habitat of *Streptococcus infantarius* subsp. *infantarius*. *Int J Food*  
486 *Microbiol* 127:215-219.
- 487 28. Beukes EM, Bester BH, Mostert JF (2001) The microbiology of South African traditional  
488 fermented milks. *Int J Food Microbiol* 63:189-197.
- 489 29. Ouadghiri M, Vancaneyt M, Vandamme P, Naser S, Gevers D, Lefebvre K, Swings J, Amar M  
490 (2009) Identification of lactic acid bacteria in Moroccan raw milk and traditionally fermented  
491 skimmed milk 'Iben'. *J App Microbiol* 106:486-495.
- 492 30. Tamang JP, Thapa N, Tamang B, Rai A, Chettri R (2014) Lactic acid bacteria in Fermented Foods  
493 and Beverages. In *Health Benefits of Fermented Foods and Beverages*, Tamang JP (ed), CRC Press,  
494 USA, pp 1–87.
- 495 31. Garcia-Caquela T, Tabasco R, Pelaez C, Requena T (2009) Simultaneous detection and  
496 enumeration of viable lactic acid bacteria and *bifidobacteria* in fermented milk by using propidium  
497 monoazide and real-time PCR. *Int Dairy J* 19:405-409.
- 498 32. Gueimonde M, Delgado S, Mayo B, Ruas-Madiedo P, Margolles A, Reyes-Gavilan CG (2004)  
499 Viability and diversity of probiotic *Lactobacillus* and *Bifidobacterium* populations included in  
500 commercial fermented milk. *Food Res Int* 37:839-850.
- 501 33. Tabasco R, Paarup T, Jenar C, Pelaez C, Requena T (2007) Selective enumeration and  
502 identification of mixed culture of *streptococcus thermophilus*, *lactobacillus delbrueckii* subsp.  
503 *Bulgarius*, *l. acidophilus*, *l. paracasei* subsp. *parasei* and *Bifidobacterium lactis* in fermented milk. *Int*  
504 *Dairy J* 17:1107-1114.

- 505 34. Mathara JM, Schillinger U, Guigar C, Franz C, Kutima P.M, Mbugua SK, Slim HK, Holzapfel WH  
506 (2008) Functional characteristics of *Lactobacillus* spp. from traditional Maasai fermented milk  
507 product in Kenya. Int J Food Microbiol. 126:57-64.
- 508 35. Sule J, Korosi T, Hucker A, Vargra L (2014) Evaluation of culture media for selective enumeration  
509 of *Bifidobacteria* and lactic acid bacteria. Brazilian J. Microbiol. 45:1023-1030.
- 510 36. Jordano R, Serrano CE, Torres M, Salmeron J (1992) Comparison of Three M17 Media for the  
511 Enumeration of *Streptococcus thermophilus* in Fermented Dairy Products. J Food Prot. 55:999-1002.
- 512 37. Khedid K, Faid M, Mokhtari A, Soulaymani A, Zinedine A (2009) Characterisation of lactic acid  
513 bacteria isolated from the one humped camel milk produced in morocco. J Microbiol Res 164:81-91.
- 514 38. Zomer A, Fernandez M, Kearney B, Fitzgerald G. F, Ventura M, van Sinderen D (2009). An  
515 interactive regulatory network controls stress response in *Bifidobacterium breve* UCC2003. J  
516 Bacteriol.191:7039-7049.
- 517 39. Devirgiliis C, Coppola D, Barile S, Colonna B, Perozzi G (2009) Characterisation of the Tn916  
518 conjugative transposon in a foodborne strain of *Lactobacillus paracasei*. App Environ Microbiol  
519 75:3866–3871.
- 520 40. Wang C.Y, Lin P.R, Ng C.C, Shyu Y.T (2010) Probiotic properties of *Lactobacillus* strains isolated  
521 from the face of breast-fed infants and Taiwanese pickled cabbage. Anaerobe 16:578–585.
- 522 41. Giri SS, Sukumaran V, Dangi NK (2012) Characteristics of Bacterial Isolates from the Gut of  
523 Freshwater Fish, *Labeo rohita* that May be Useful as Potential Probiotic Bacteria. Probiotics  
524 Antimicrob Proteins 4:238–242.
- 525 42. Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E (2006)  
526 Probiotic potential of *Lactobacillus* strains isolated from dairy products. Int Dairy J. 16:189-199.
- 527 43. Patil MM, Pal A, Anand T, Ramana KV (2010) Isolation and characterisation of lactic acid bacteria  
528 from curd and cucumber. Indian J Biotechnol 9:166-172.

- 529 44. De Vuyst L, Zamfir M, Mozzi F, Adriany T, Marshall V, Degeest B, Vaningelgem F (2003)  
530 Exopolysaccharide-producing *Streptococcus thermophilus* strains as functional starter cultures in the  
531 production of fermented milks. *Int Dairy J.* 13:707-717.
- 532 45. Oguntoyinbo FA (2014) Safety Challenges Associated with Traditional Foods of West Africa, *Food*  
533 *Rev Int* 30:338-358.
- 534 46. Owusu-Kwarteng J, Wuni A, Akabanda F, Jespersen L (2018) Prevalence and Characteristics of  
535 *Listeria monocytogenes* isolates in Raw Milk, Heated Milk and Nunu, a Spontaneously Fermented Milk  
536 Beverage, in Ghana. *Beverages* 4:1-10.
- 537 47. Kaktcham PM, Zambou NF, Fonteh AF, Sieladie DV, Tchouanguep MF (2011) Characterisation of  
538 bacteriocin produced by *Lactobacillus rhamnosus* 1K isolated from traditionally fermented milk in  
539 the western highlands region of Cameroon. *N Y Sci J* 4:121-128.
- 540 48. Heredia-Castro PY, Méndez-Romero JI, Hernández-Mendoza A, Acedo-Félix E, González-Córdova  
541 AF, Vallejo-Cordoba B (2015) Antimicrobial activity and partial characterization of bacteriocin-like  
542 inhibitory substances produced by *Lactobacillus* spp. isolated from artisanal Mexican cheese. *J Dairy*  
543 *Sci* 98:8285-8293.
- 544 49. Banwo K, Sanni AK, Tan H (2012) Technological properties and probiotic potential of  
545 *Enterococcus faecium* strains isolated from cow milk. *J Applied Microbiol* 114: 229-241.
- 546 50. de Lima MDSF, da Silva RA, da Silva MF, da Silva PAB, Costa RMPB, Teixeira JAC, Porto ALF,  
547 Cavicant MTH (2018) Brazilian kefir-fermented sheep's milk, a source of antimicrobial and  
548 antioxidant peptides. *Probiotics Antimicrob Proteins* 10:446–455.
- 549 51. Sharma C, Brij PS, Nishchal T, Sachin G, Sanjolly G, Santosh KM, Harsh P (2017) Antibacterial  
550 effects of *Lactobacillus* isolates of curd and human milk origin against food-borne and human  
551 pathogens. *3 Biotech*, 7:31.

552 52. Mantel ES, Sakyi-Dawson E, Amoa-Awua WK (2003) Antimicrobial interactions of microbial  
553 species involved in the fermentation of cassava dough into agbelima with particular reference to the  
554 inhibitory effect of lactic acid bacteria on enteric pathogens. *Int J Food Microbiol* 89:41-50.

555 53. Amoa-Awua WKA, Owusu M, Feglo P (2005) Utilization of unfermented cassava flour for the  
556 production of an indigenous African fermented food, agbelima. *World J. Microbiol. Biotechnol.*  
557 20:1201-1207.

558 54. Owusu-Kwarteng J, Tano-Debrah K, Akabanda F, Jespersen L (2015) Technological properties and  
559 probiotic potential of *Lactobacillus fermentum* strains isolated from West African fermented millet  
560 dough. *BMC Microbiol* 15:261.

561 55. Fricourt BV, Barefoot SF, Testin RF, Hayasaka SS (1994) Detection and activity of plantaricin F, an  
562 antibacterial substance from *Lactobacillus plantarum* BF001 isolated from processed channel catfish.  
563 *J Food Protection*. 57: 698-702.

564 56. Piard JC, Desmazeand M (1991) Inhibiting factors produced by lactic acid bacteria. Oxygen  
565 metabolites and catabolism end products. *Le Lait*. 71:525-541.

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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, <i>pheS</i> , <i>rpoA</i> gene sequencing
1	LO1	A	<i>Lactobacillus fermentum</i>
2	LO1	B	<i>Lactobacillus fermentum</i>
33	LO1	B	<i>Lactobacillus fermentum</i>
35	LO1	B	<i>Lactobacillus fermentum</i>
54	LO1	B	<i>Lactobacillus fermentum</i>
36	LO1	D	<i>Lactobacillus fermentum</i>
37	LO1	D	<i>Lactobacillus fermentum</i>
69	LO1	G	<i>Streptococcus thermophilus</i>
71	LO1	G	<i>Streptococcus thermophilus</i>
74	LO1	G	<i>Streptococcus thermophilus</i>
70	LO1	H	<i>Streptococcus thermophilus</i>
73	LO1	H	<i>Streptococcus thermophilus</i>
72	LO1	H	<i>Streptococcus thermophilus</i>
34	LO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
53	LO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
17	LO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
5	LO2	A	<i>Lactobacillus fermentum</i>
19	LO2	A	<i>Lactobacillus fermentum</i>
3	LO2	B	<i>Lactobacillus fermentum</i>
38	LO2	B	<i>Lactobacillus fermentum</i>
55	LO2	B	<i>Lactobacillus fermentum</i>
56	LO2	D	<i>Lactobacillus fermentum</i>
57	LO2	D	<i>Lactobacillus fermentum</i>
75	LO2	G	<i>Streptococcus thermophilus</i>
76	LO2	H	<i>Streptococcus thermophilus</i>
4	LO2	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
18	LO2	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
39	LO2	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
58	LO3	A	<i>Lactobacillus fermentum</i>
7	LO3	A	<i>Lactobacillus fermentum</i>
20	LO3	A	<i>Lactobacillus fermentum</i>
40	LO3	A	<i>Lactobacillus fermentum</i>
41	LO3	A	<i>Lactobacillus fermentum</i>
59	LO3	A	<i>Lactobacillus fermentum</i>
6	LO3	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
8	LO3	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
21	LO4	B	<i>Lactobacillus fermentum</i>
61	LO4	B	<i>Lactobacillus fermentum</i>
<b>9</b>	LO4	E	<i>Leuconostoc pseudomesenteroides</i>
<b>42</b>	LO4	E	<i>Leuconostoc pseudomesenteroides</i>
<b>43</b>	LO4	F	<i>Lactobacillus senioris</i>

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

578 EO1 and EO2: Production sites from Eke.

**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, <i>pheS</i> , <i>rpoA</i> gene sequencing
79	LO4	I	<i>Streptococcus infantarius</i>
80	LO4	I	<i>Streptococcus infantarius</i>
77	LO4	I	<i>Streptococcus infantarius</i>
78	LO4	I	<i>Streptococcus infantarius</i>
10	LO4	I	<i>Streptococcus infantarius</i>
44	LO4	I	<i>Streptococcus infantarius</i>
22	LO4	J	<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i>
60	LO4	J	<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i>
46	LO5	A	<i>Lactobacillus fermentum</i>
47	LO5	A	<i>Lactobacillus fermentum</i>
12	LO5	A	<i>Lactobacillus fermentum</i>
82	LO5	G	<i>Streptococcus thermophilus</i>
88	LO5	G	<i>Streptococcus thermophilus</i>
86	LO5	H	<i>Streptococcus thermophilus</i>
81	LO5	H	<i>Streptococcus thermophilus</i>
87	LO5	H	<i>Streptococcus thermophilus</i>
83	LO5	I	<i>Streptococcus infantarius</i>
84	LO5	I	<i>Streptococcus infantarius</i>
85	LO5	I	<i>Streptococcus infantarius</i>
11	LO5	J	<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i>
23	LO5	J	<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i>
24	LO5	J	<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i>
45	LO5	J	<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i>
14	LO6	A	<i>Lactobacillus fermentum</i>
63	LO6	A	<i>Lactobacillus fermentum</i>
13	LO6	C	<i>Lactobacillus fermentum</i>
25	LO6	C	<i>Lactobacillus fermentum</i>
26	LO6	C	<i>Lactobacillus fermentum</i>
48	LO6	C	<i>Lactobacillus fermentum</i>
50	LO6	C	<i>Lactobacillus fermentum</i>
62	LO6	C	<i>Lactobacillus fermentum</i>
92	LO6	G	<i>Streptococcus thermophilus</i>
89	LO6	G	<i>Streptococcus thermophilus</i>
91	LO6	G	<i>Streptococcus thermophilus</i>
90	LO6	H	<i>Streptococcus thermophilus</i>
49	LO6	J	<i>Lactobacillus delbrueckii</i> subsp. <i>indicus</i>
28	EO1	A	<i>Lactobacillus fermentum</i>
29	EO1	A	<i>Lactobacillus fermentum</i>
51	EO1	A	<i>Lactobacillus fermentum</i>
64	EO1	A	<i>Lactobacillus fermentum</i>

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

581 EO1 and EO2: Production sites from Eke

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**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, <i>pheS</i> , <i>rpoA</i> gene sequencing
95	EO1	I	<i>Streptococcus infantarius</i>
30	EO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
65	EO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
27	EO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
94	EO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
93	EO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
15	EO1	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
31	EO2	A	<i>Lactobacillus fermentum</i>
32	EO2	A	<i>Lactobacillus fermentum</i>
68	EO2	A	<i>Lactobacillus fermentum</i>
67	EO2	F	<i>Lactobacillus senioris</i>
97	EO2	G	<i>Streptococcus thermophilus</i>
96	EO2	G	<i>Streptococcus thermophilus</i>
99	EO2	G	<i>Streptococcus thermophilus</i>
100	EO2	G	<i>Streptococcus thermophilus</i>
98	EO2	G	<i>Streptococcus thermophilus</i>
16	EO2	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
66	EO2	J	<i>Lactobacillus delbrueckii subsp. indicus</i>
52	EO2	K	<i>Enterococcus thailandicus</i>

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

585 EO1 and EO2: Production sites from Eke

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

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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	<i>Enterococcus thailandicus</i>	6.33 ± 0.08 <sup>bc</sup>	6.18 ± 0.20 <sup>bc</sup>	6.31 ± 0.04 <sup>bc</sup>	2.90 ± 0.26 <sup>e</sup>	7.68 ± 0.07 <sup>ab</sup>	7.75 ± 0.09 <sup>ab</sup>	7.55 ± 0.10 <sup>ab</sup>	7.61 ± 0.11 <sup>ab</sup>
11	<i>Lactobacillus delbrueckii subsp. indicus</i>	6.68 ± 0.04 <sup>b</sup>	5.35 ± 0.06 <sup>c</sup>	5.87 ± 0.07 <sup>bc</sup>	4.26 ± 0.03 <sup>d</sup>	5.60 ± 0.07 <sup>bc</sup>	5.73 ± 0.14 <sup>bc</sup>	5.56 ± 0.14 <sup>bc</sup>	5.23 ± 0.09 <sup>c</sup>
13	<i>Lactobacillus fermentum</i>	7.85 ± 0.00 <sup>ab</sup>	7.48 ± 0.02 <sup>ab</sup>	7.47 ± 0.01 <sup>ab</sup>	7.32 ± 0.00 <sup>b</sup>	8.29 ± 0.05 <sup>a</sup>	8.29 ± 0.35 <sup>a</sup>	8.25 ± 0.10 <sup>a</sup>	7.18 ± 0.81 <sup>b</sup>
43	<i>Lactobacillus senioris</i>	7.78 ± 0.13 <sup>ab</sup>	7.68 ± 0.06 <sup>ab</sup>	7.62 ± 0.02 <sup>ab</sup>	5.98 ± 0.19 <sup>bc</sup>	7.59 ± 0.12 <sup>ab</sup>	7.65 ± 0.05 <sup>ab</sup>	7.66 ± 0.03 <sup>ab</sup>	7.69 ± 0.02 <sup>ab</sup>
9	<i>Leuconostoc pseudomesenteroides</i>	6.56 ± 0.15 <sup>b</sup>	6.12 ± 0.02 <sup>bc</sup>	6.33 ± 0.16 <sup>bc</sup>	-	7.94 ± 0.14 <sup>ab</sup>	7.29 ± 0.16 <sup>b</sup>	7.96 ± 0.17 <sup>ab</sup>	6.39 ± 0.16 <sup>bc</sup>
10	<i>Streptococcus infantarius</i>	6.86 ± 0.04 <sup>b</sup>	6.46 ± 0.11 <sup>b</sup>	6.38 ± 0.21 <sup>bc</sup>	-	8.18 ± 0.47 <sup>a</sup>	7.84 ± 0.01 <sup>ab</sup>	7.70 ± 0.07 <sup>ab</sup>	7.47 ± 0.03 <sup>ab</sup>
73	<i>Streptococcus thermophilus</i>	5.94 ± 0.03 <sup>bc</sup>	4.51 ± 0.03 <sup>c</sup>	4.37 ± 0.18 <sup>d</sup>	-	6.32 ± 0.06 <sup>bc</sup>	5.26 ± 0.07 <sup>c</sup>	5.40 ± 0.08 <sup>bc</sup>	2.86 ± 0.10 <sup>e</sup>

599 Data represent the mean of the viable count in two experiments expressed as mean ± standard deviation in log<sub>10</sub> Cfu/ml.

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

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