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Genotypic characterization and safety assessment of lactic acid bacteria from indigenous African fermented food products

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Abstract

Background: Indigenous fermented food products play an essential role in the diet of millions of Africans. Lactic acid bacteria (LAB) are among the predominant microbial species in African indigenous fermented food products and are used for different applications in the food and biotechnology industries. Numerous studies have described antimicrobial susceptibility profiles of LAB from different parts of the world. However, there is limited information on antimicrobial resistance profiles of LAB from Africa. The aim of this study was to characterize 33 LAB previously isolated from three different African indigenous fermented food products using (GTG)₅-based rep-PCR, sequencing of the 16S rRNA gene and species-specific PCR techniques for differentiation of closely related species and further evaluate their antibiotic resistance profiles by the broth microdilution method and their haemolytic activity on sheep blood agar plates as indicators of safety traits among these bacteria.

Results: Using molecular biology based methods and selected phenotypic tests such as catalase reaction, CO₂ production from glucose, colonies and cells morphology, the isolates were identified as Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus ghanensis, Lactobacillus plantarum, Lactobacillus salivarius, Leuconostoc pseudomesenteroides, Pediococcus acidilactici, Pediococcus pentosaceus and Weissella confusa. The bacteria were susceptible to ampicillin, chloramphenicol, clindamycin and erythromycin but resistant to vancomycin, kanamycin and streptomycin. Variable sensitivity profiles to tetracycline and gentamicin was observed among the isolates with Lb. plantarum, Lb. salivarius, W. confusa (except strain SK9-5) and Lb. fermentum strains being susceptible to tetracycline whereas Pediococcus strains and Lb. ghanensis strains were resistant. For gentamicin, Leuc. pseudomesenteroides, Lb. ghanensis and Ped. acidilactici strains were resistant to 64 mg/L whereas some W. confusa and Lb. plantarum strains had a MIC value of 16 mg/L and 32 mg/L respectively. No β-haemolytic activity was observed, however, α-haemolytic activity was observed in 27% (9) of the strains comprising Lb. salivarius (6), W. confusa (2) and Lb. delbrueckii (1) isolates.

Conclusions: The resistance to kanamycin and vancomycin is probably an intrinsic feature since similar observations were reported in the literature for LAB. Low prevalence of pathogenicity indicator traits were observed among the isolates especially with the presence of poor haemolytic activities and they could therefore be considered as interesting candidates for selection of starter cultures or probiotics for different applications.
Background

Fermented food products have a long history and form significant part of the diet of many indigenous communities in the developing world [1-3]. African indigenous fermented food products, like many fermented food products in different parts of the world are deemed to have improved flavour, texture, increased shelf-life, bioavailability of micronutrients, and reduced or absence of anti-nutrition and toxic compounds among others [4-7]. Previous works on African fermented foods have revealed a complex and significant microbial biodiversity responsible for these inherent desirable characteristics [6,8-12] and Lactobacillus, Leuconostoc and to a lesser extent Pediococcus, Lactococcus and Weissella species are the most predominant LAB genera [4,13].

Some of these foods include; lafun, kenkey, koko, dawadawa/soumbala, nyarmie, garis, agbelima and pito/dolo [9,11,14-17]. Koko is a thick porridge which is made from millet, corn or sorghum and is consumed in many communities in Ghana. According to Lei and Jacobsen [4], the predominant microbial species in koko sour water (KSW) obtained from millet were W. confusa, Lb. fermentum, Lb. salivarius and Pediococcus spp. Pito is also a fermented alcoholic beverage which is popular but in different variants among many indigenous communities in sub-Sahara African countries such as Burkina Faso, Ghana, Togo, Nigeria, and Benin among others. It is produced from malted sorghum or maize and sometimes a combination of both. The production process involves milling of malted sorghum, mashing, acidification, cooking, cooling, and alcoholic fermentation of the wort by the back-slopping process which involves using yeasts from previously fermented product [9,18]. It is therefore a spontaneous mixed fermentation product in which the predominant microbial florae are yeasts and LAB. Lb. fermentum, Lb. delbrueckii and Pediococcus species are the predominant LAB species [9,18].

Cocoa is arguably the most significant cash crop in many tropical countries such as Ivory Coast and Ghana. Raw cocoa beans are embedded in mucilaginous pulp and characterized by an astrangent and unpleasant taste and flavour. To obtain the characteristics cocoa flavour, the mucilaginous cocoa pulp has to be fermented, dried and then roasted [8]. Cocoa fermentation is therefore the main stage in cocoa post-harvest processing [19] and contributes significantly to the characteristics final flavour of chocolates. There is microbial succession in the natural or spontaneous fermentation process of cocoa with LAB being among the dominant microbial species [8,19].

LAB are very significant in the dairy and biotechnology industries. They are used as starter cultures for dairy fermented food products, human and animal health products and animals feed inoculants. They have been classified as ‘generally recognized as safe’ (GRAS) due to their general occurrence in many fermented and non-fermented food products and also being part of the human commensal micro-flora. There have however been a few reported cases on clinical infections such as endocarditis, bacteraemia, and urinary tract infections caused by these microbial species, though in all these cases, patients had underlying conditions which predisposed them to infections particularly in the case of endocarditis [20,21]. Lactobacillus rhamnosus, Lactococcus lactis, Leuconostoc species and Lactobacillus casei (paracasei) have been cited in some non-enterococcal LAB endocarditis cases [20]. In view of this, it is relevant to have a more thorough safety assessment of LAB before their uses as live cultures for varying applications in the food and feed industry.

Moreover, the widespread use of antibiotics in human medicines and farm practices has over the past century led to the spread of antibiotic resistant microorganisms. Antibiotics efficacy on bacteria is defined in terms of their MIC (mg/L) value which is considered as the reference point for comparing different antibiotics potency [22]. It has been shown that genes coding for antibiotics resistance can be transferred among bacteria of different genera and thus to pathogenic bacteria which consequently cannot be treated with previously successful antibiotics [23]. In a study by Temmerman et al. [24], it was observed that out of a total of 268 bacteria isolated from 55 European probiotics products, antibiotic resistance among 187 of the isolates was detected against kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillin G (23%), erythromycin (16%) and chloramphenicol (11%) whereas 68.4% of the isolates showed resistance against multiple antibiotics including intrinsic resistances. According to Kastner et al. [25], out of 200 starter cultures and probiotic bacteria isolated from 90 different food sources in Zurich, 27 isolates exhibited resistance patterns that could not be ascribed as an intrinsic feature of the respective genera. Ninety four tetracycline-resistant LAB strains from fermented dry sausages were also reported by Gevers et al. [26] in which it was attributed to the presence of tetracycline resistance tet(M) gene.

While many studies have investigated the resistance profiles of LAB from the European origin [27-29], much less have been reported on the antimicrobial susceptibility of LAB of African origin. In some developing countries for instance, there is influx of antibiotics from different parts of the world into the market and subsequently, stricter regulations and laws are not enforced to regulate antibiotics uses as human medicine [30,31]. Antibiotics could even be purchased from local pharmacies as over-the-counter preparations, without prescriptions [32]. In Ghana, clinical isolates
with multiple drug resistance to the four predominantly used antibiotic drugs; ampicillin, cotrimoxazole, tetracycline and chloramphenicol have been reported [33]. Ouoba et al. [34] also observed that probiotic LAB and bifidobacteria of African and European origin were resistant to vancomycin, tetracycline, kanamycin, sulphamethoxazole, neomycin, nalidixan, apramycin and colistin. Thus the potential health risks that could result from the transfer of antibiotic resistance genes from LAB reservoir strains to bacteria in the resident microflora of the human gastrointestinal tract or pathogenic bacteria cannot be overlooked especially if the strains are to be introduced as live culture in food or feed products. To prevent the spread of antibiotics resistant genes, an application for European Food Safety Authority (EFSA) approval of microorganisms as feed additives or plant protection agents for instance, requires mandatory information on frequently used drugs resistant profiles of the bacteria [35]. Inter-genus and inter-species differences exist in antimicrobial susceptibility of bacteria as it has been indicated in some studies [29,34]. Genotyping of microbial species and their safety evaluations are hence essential in the microbiological risk assessment process prior to further study of these bacteria for different applications in the food and feed industry.

The aim of the present study was to genotypically characterise 33 LAB isolated from African indigenous fermented food products and further evaluate their safety characteristics in terms of resistance to relevant antibiotics and haemolytic activities in order to increase our present limited knowledge on antibiotic resistance profiles of LAB from African indigenous fermented food products.

**Methods**

**Bacterial strains, cultivation conditions and preliminary phenotypic characterizations**

The lactic acid bacteria strains used in this study were obtained from three different African indigenous fermented foods (Table 1). Stock-cultures were maintained in MRS broth (Oxoid Ltd., CM0359, pH 6.2 ± 0.2, Basingstoke, Hempshire, England) supplemented with 20% glycerol and stored at −80°C. Working cultures were made by inoculating 10 ml MRS broth with freeze-stock culture and then incubated at 37°C overnight in a standard incubator without agitation. The isolates were characterized by colony morphology and cells morphology using phase-contrast microscopy, CO₂ production from glucose in MRS broth with Durham tubes and catalase reaction with 3% H₂O₂.

**Genotypic characterization**

**Genomic DNA preparation for PCR and sequencing reactions**

Overnight-culture of each strain was streak-plated on MRS agar (Oxoid Ltd., CM0361, pH 6.2 ± 0.2, Basingstoke, Hempshire, England) and incubated at 37°C overnight in a standard incubator without agitation. The isolates were characterized by colony morphology and cells morphology using phase-contrast microscopy, CO₂ production from glucose in MRS broth with Durham tubes and catalase reaction with 3% H₂O₂.

### Table 1 Sources of isolation of 33 lactic acid bacteria investigated in this study

| Species and strains | Source of isolation | Raw materials used | Reference |
|---------------------|---------------------|--------------------|-----------|
| *Lb. plantarum*     | Fermenting cocoa beans (FCB) | Cocoa pulp⁴ | [8]       |
| L106, L547, L544, L415, L263, L260, L142, LA113 | | | |
| *L. plantarum*      | Koko sour water (KSW) | Sorghum, maize, millet⁵ | [14] |
| S1, S2              | | | |
| *L. ghanensis*      | FCB                 | a                  | [8]       |
| L489, L499          | | | |
| *Leuc. pseudomesenteroides* | FCB | a | [8] |
| L8                  | | | |
| *Lb. fermentum*     | Dolo and pito wort (DPW) | Sorghum, maize⁶ | [9] |
| ZN7b-2, ZN7b-7      | | | |
| *Lb. delbrueckii*   | DPW                 | c                  | [9]       |
| species             | | | |
| ZN7a-9              | | | |
| *Lb. salivarius*    | KSW                 | b                  | [14]      |
| FK10-10, FK11-2, FK11-4, FK11-8, FK11-9 | | | |
| *Ped. acidilactici* | KSW                 | b                  | [14]      |
| N8, N9, N10         | | | |
| *Ped. pentosaceus*  | KSW                 | b                  | [14]      |
| P4, P5, S4          | | | |
| *W. confusa*        | KSW                 | b                  | [14]      |
| P2, P3, SK9-2, SK9-5, SK9-7, FK10-9 | | | |
kit (Bio-Rad, Hercules, CA, USA) and following the manufacturer's instructions. DNA was stored at −20°C and used for all PCR reactions mentioned in this study.

Rep-PCR
Genomic DNA was analysed with the rep-PCR fingerprinting method using the GTG<sub>3</sub> (5’-AGA GTT TGA TYM TGG CTC AG-3’) and 1510r (5’-ACG GYT ACC TTT TTA CGA CTT-3’) primer (DNA Technology A/S, Denmark) with the protocol of Nielsen et al. [21]. Electrophoresis conditions and image analysis with the Bionumerics software package (Applied Maths, Sint-Martens-Latem, Belgium) were performed as previously [8].

16S rRNA gene sequencing
PCR amplification of 16S rRNA gene of all the isolates was performed with the primers 7f (5’-AGA GTT TGA TYM TGG TGC AG-3’) and 1510r (5’-ACG GYT ACC TTT TTA CGA CTT-3’) [36] (DNA Technology A/S, Denmark). The reaction mixture consisted; 50 μl of 10X PCR reaction buffer (Fermentas, Germany), 0.2 mM dNTP-mix (Fermentas, Germany), 1.5 mM MgCl<sub>2</sub>, 0.1 pmol/μl primers 7f and 1510r, 0.5 μl formamide (Merck), 0.5 μl of 1 mg/ml bovine serum albumin (New England Biolabs), 0.25 μl DreamTaq DNA polymerase (5 u/μl) (Fermentas, Germany) and 1.5 μl of the extracted genomic DNA. The volume of the PCR mixture was adjusted to 50 μl with sterile MilliQ water. PCR amplification was performed in DNA thermocycler (Gene Amp PCR System 2400, Perkin Elmer) at the following thermocycling conditions: 5 min of initial denaturation at 94°C, followed by 30 cycles of 94°C for 90 seconds, 52°C for 30 seconds, 72°C for 90 seconds and a final elongation step of 72°C for 7 minutes. To check for successful PCR amplification, 10 μl of the PCR product was electrophoresed in a 2% agarose gel in 1X TBE (1 hr, 100 V). PCR products were purified of DNA amplification reagents using NucleoSpin<sup>®</sup> DNA purification kit by following the manufacturer’s instructions. Sequencing was performed in both directions with the universal primers 27f (5’-AGA GTT TGA TYM TGG CTC AG-3’) and 1492r (5’-TAC GGY TAC CTT GGT ACC ACT T-3’) by a commercial sequencing facility (Macrogen Inc., Korea). The sequences were corrected using Chromas version 2.33 (Technelysium Pty Ltd). Corrected sequences were aligned to 16S rRNA gene sequences in the GenBank database using the BLAST algorithm [37].

Differentiation of Lactobacillus plantarum, Lb. paraplanatarum and Lb. pentosus by multiplex PCR using recA gene-based primers
A multiplex PCR assay for differentiation of Lb. plantarum, Lb. paraplanatarum and Lb. pentosus was performed as described by Torriani et al. [38]. Genomic DNA from Lb. paraplanatarum LTH5200, Lb. pentosus DSM20314<sup>T</sup> and Lb. plantarum DSM20174<sup>T</sup> were used as positive control and genomic DNA from Leuconostoc pseudomesenteroides L8 and Lb. ghanensis L499 were used as negative control.

Differentiation of Weissella confusa and W. cibaria strains
The closely related species W. confusa and W. cibaria were differentiated from each other by a W. confusa species-specific PCR method as described by Fusco et al. [39]. Genomic DNA from W. confusa DSM11983<sup>T</sup> was used as positive control. Genomic DNA from the following species was used as negative control; W. cibaria 17699<sup>T</sup>, Pediococcus acidilactici DSM20284<sup>T</sup>, Ped. pentosaceus DSM20336<sup>T</sup>, Lb. fermentum DSM20052<sup>T</sup>, Lb. pentosus DSM20314<sup>T</sup>, Lb. paraplanatarum LTH5200, Lb. delbrueckii subsp. lactis DSM20073, and Lb. delbrueckii subsp. bulgaricus DSM20080.

Safety characterizations
Antibiotics MIC testing by the broth microdilution method
Nine antibiotics were included in the assay: ampicillin and vancomycin as inhibitors of cell wall synthesis, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin and tetracycline as inhibitors of protein synthesis. All antibiotics were obtained from Sigma (St. Louis, Mo., USA) in powdered form and 2 g/L stock solutions prepared. Chloramphenicol and erythromycin stock solutions were prepared in 95% ethanol and the remaining antibiotics stock solutions prepared in sterile MilliQ water and filter sterilized (MILLLEX GP Syringe Driven Filter Unit, 0.22 μm, Millipore, Ireland). Aliquots (1 ml) of the stock solutions were stored at −20°C. The minimum inhibitory concentration of antibiotics (MICs, mg/L) for all bacteria (except Lb. ghanensis L489 and Lb. delbrueckii ZN9<sub>7</sub>) was determined by a modification of the broth micro-dilution method reported by Mayrhofer et al. [40] and Domig et al. [41] with different antibiotics concentration ranges depending on the particular antibiotic. In summary of the method, antibiotics stock solution (2.0 g/L) was added to MRS broth (pH 6.2) and then followed by log<sub>2</sub> serial dilutions to obtain the appropriate antibiotics concentrations. The media (198 μl) with the appropriate antibiotic concentration was then dispensed into wells of sterile commercial flat-bottom microtitre plates with lids (Fisher Scientific, Biotech Line A/S, Denmark) and stored at −20°C for overnight. Prior to inoculation, the plates were allowed to attain room temperature. Inocula were prepared by suspending single isolated colonies of bacteria (MRS-agar, 37°C, 48 hrs) in 3 ml sterile 0.9% NaCl. Turbidity of the cells suspension was adjusted to 1 McFarland standard equivalent (approx. 3x10<sup>8</sup>cfu/ml). The plates were inoculated with 2 μl of the cell suspension to obtain approximately 3x10<sup>6</sup> cfu/ml in each well. Plates were
incubated under anaerobic conditions at 37°C for 24 hrs (COY Laboratory Products INC, USA). All MIC testing was performed in duplicates and with one antibiotic free well inoculated and an un-inoculated well containing test media as negative control. The MIC was defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison with inoculated and un-inoculated antibiotic-free wells.

Haemolysis test
The bacteria were tested for haemolysis on tryptone soy agar with sheep blood (TSA-SB) (Oxoid Ltd, PB3012A, pH 7.5 ± 0.2, Wesel, Germany) by streaking 24 hr cultures on the blood agar plates followed by incubation at 37°C under anaerobic conditions (Anaerogen, Oxoid) for 24 hrs. The appearance of clear zones around the bacteria colonies indicated the presence of β-haemolysis whereas green zones around the colonies suggested α-haemolysis [42].

Nucleotide accession numbers
The nucleotide sequences determined in this study have been assigned GenBank Accession Nos. JQ801703-JQ801728.

Results
Genotypic characterization
The LAB included in the study (Table 1) were isolated from three different African indigenous fermented food products. To confirm their identities, selected phenotypic tests such as catalase reaction, CO₂ production from glucose, colony and cell morphology along with genotypic identification methods were performed. Initially all 33 strains were subjected to rep-PCR (GTG)₅ fingerprinting technique for genotypic grouping. Numerical analysis of the (GTG)₅-PCR fingerprint band patterns obtained is shown in Figure 1. However, re-sequencing of the 16S rRNA gene indicated that strains S1 and S2 have high level of sequence homology to both Lb. paraplanatarum and Lb. plantarum. A multiplex PCR assay using species-specific primers targeting the recA gene was used to achieve unambiguous identification of all strains belonging to the Lb. plantarum-group by 16S rRNA gene sequencing (Figure 2). All these strains including strains S1 and S2 produced a PCR product of size 318 bp similar to the Lb. plantarum DSM20174ᵀ positive control strain and were consequently confirmed to be Lb. plantarum strains.

Also, using the W. confusa species-specific PCR technique reported by Fusco et al. [39], PCR amplified products were obtained for all the strains with high 16S rRNA gene similarity to both W. confusa and W. cibaria as shown in Figure 3. The size of the amplicon (225 bp) obtained for each of the strains was similar to that obtained for W. confusa LMG 11983ᵀ which was used as reference strain. This therefore confirms that the strains; P2, P3, SK9-2, SK9-5, SK9-7 and FK10-9 were W. confusa strains. In the previous study [9], strains ZN7a-9, ZN7b-2 and ZN7b-7 were identified as Lb. delbrueckii strains based on ITS-PCR/RFLP analysis and PFGE-Asc I fingerprint patterns. However, a BLAST search of the sequences of ZN7b-2 and ZN7b-7 in the GenBank database gave high identity values for Lb. fermentum strains. As also shown in the dendrogram of the rep-PCR fingerprint band patterns, these two strains also formed one cluster which was separated from ZN7a-9 which sequence has high similarity value to Lb. delbrueckii sequences in the GenBank database. Thus ZN7b-2 and ZN7b-7 were re-identified as Lb. fermentum strains.

Antibiotic susceptibility testing
The results of antibiotic susceptibility testing are shown in Table 2. The bacteria were considered resistant to a particular antibiotic when the MIC (mg/L) values obtained were higher than the recommended breakpoint value defined at species level by the FEEDAP Panel; Panel on Additives and Products or Substances used in Animal Feed [22]. All strains were resistant to kanamycin (MIC range 8–128 mg/L), streptomycin (64–128 mg/L) and vancomycin (MIC range 0.5-8.0 mg/L) within the MIC ranges assayed (Table 2). The strains were highly susceptible to ampicillin (0.5-2.0 mg/L), chloramphenicol (2–4 mg/L), clindamycin (0.5-2.0 mg/L) and erythromycin (0.5-1.0 mg/L). The chloramphenicol MIC value (4 mg/L) obtained for Lb. plantarum, Lc. pseudomesenteroides, Lb. ghanensis and Lb. fermentum was one-fold higher than the MIC value obtained for Ped. acidilactici, Ped. pentosaceus and Weissella species. Lb.
plantarum, Lb. salivarius, W. confusa (except strain SK9-5) and Lb. fermentum strains were susceptible to tetracycline. However, Pediococcus strains and the Lb. ghanensis strain were resistant to tetracycline since the MIC values (16–32 mg/L) obtained were higher than the recommended breakpoint value (8 mg/L). The resistance profile of the strains to gentamicin varies at both species and strains level. Leuc. pseudomesenteroides, Lb. ghanensis and Ped. acidilactici strains were resistant to 64 mg/L gentamicin. However, the majority (4 out of 5) of W. confusa strains have MIC value of 16 mg/L whereas the MIC value obtained for most (7 strains) of Lb. plantarum strains was 32 mg/L.
Haemolysis testing
After streaking the bacteria on tryptone soy agar with sheep blood, no β-haemolysis was observed in any of the bacteria strains. However, as shown in Figure 4, α-haemolysis was observed in 9 out of the 33 strains of which 6 strains were Lb. salivarius, 2 strains W. confusa and the Lb. delbrueckii species strain.

Discussion
The reproducibility and discriminatory power of rep-PCR (GTG)$_5$ in typing at species and subspecies level have previously been reported [8,43-45] and also in the present study the technique proved useful for genotypic fingerprinting and grouping.

Lb. plantarum, Lb. paraplanatarum and Lb. pentosus share very similar 16S rRNA gene sequences; ≥ 99% and also have similar phenotypic traits making it difficult to differentiate these three species [38]. The recA gene sequence was therefore considered a reliable and useful target in order to differentiate Lb. plantarum, Lb. pentosus and Lb. paraplanatarum species [38]. In this study, the size of the amplicons of all the 10 presumptive Lb.

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**Figure 2** Amplification product obtained from recA multiplex PCR assay. Lane labelled S; 1 kb ladder from Fermentas, Lane 1, 2 and 3, PCR amplification products from Lb. paraplanatarum LTH 5200$^T$, Lb. pentosus DSM 20314$^T$ and Lb. plantarum subsp. plantarum DSM 20174$^T$ respectively. Lane 4, 5, 6, LA113, 7, Leuc. pseudomesenteroides L8 (negative control), 8, L142, 9, L106, 10, L260, 11, L415, 12, L263, 13, L547, 14, L544, 15, L499 (negative control), 16, MillQ water (control). DNA from negative control strains was not amplified. Lane numbers are indicated in bold.

**Figure 3** W. confusa species-specific PCR assay. Lane labelled S; 1 kb ladder from Fermentas, 1; sterile MilliQ water (control), lane 2 and 3, W. cibaria LMG 17699$^T$ and W. confusa LMG 11983$^T$, Lane 4; P2, 5, P3, 6, SK9-2, 7, FK11-9, 8, SK9-7, 9, SK9-5, 10, Ped. acidilactici DSM 20284$^T$, 11; Ped. pentosaceus DSM 20336$^T$, 12, Lb. fermentum DSM 20052$^T$, 13, Lb. pentosus DSM 20314$^T$, 14, Lb. paraplanatarum LTH 5200$^T$, 15, Lb. delbrueckii subsp. lactis DSM 20073, 16, Lb. delbrueckii subsp. bulgaricus DSM 20080. Lane numbers are indicated in bold.
| Antibiotic | Species          | n   | Number of strains with MIC (mg/L): |
|------------|------------------|-----|-----------------------------------|
|            |                  |     | 0.25     | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |
| AMP        | Lb. plantarum    | 10  | 10       |     |   |   |   |    |    |    |    |    |
|            | Leuc. pseudomesenteroides | 1 | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. ghanensis    | 1   | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. fermentum    | 2   | 2         |     |   |   |   |    |    |    |    |    |
|            | Lb. salivarius   | 6   | 6         |     |   |   |   |    |    |    |    |    |
|            | Ped. acidilactici | 3 | 2         | 1   |    |   |   |    |    |    |    |
|            | W. confusa       | 5   | 5         |     |   |   |   |    |    |    |    |    |
|            | Ped. pentosaceus | 3   | 2         | 1   |    |   |   |    |    |    |    |
| CHL        | Lb. plantarum    | 10  | 10       |     |   |   |   |    |    |    |    |    |
|            | Leuc. pseudomesenteroides | 1 | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. ghanensis    | 1   | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. fermentum    | 2   | 2         |     |   |   |   |    |    |    |    |    |
|            | Lb. salivarius   | 6   | 6         |     |   |   |   |    |    |    |    |    |
|            | Ped. acidilactici | 3 | 3         |     |    |   |   |    |    |    |    |
|            | W. confusa       | 5   | 5         |     |   |   |   |    |    |    |    |    |
|            | Ped. pentosaceus | 3   | 3         |     |    |   |   |    |    |    |    |    |
| CLIN       | Lb. plantarum    | 10  | 8         | 1   | 1 |    |   |    |    |    |    |    |
|            | Leuc. pseudomesenteroides | 1 | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. ghanensis    | 1   | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. fermentum    | 2   | 2         |     |   |   |   |    |    |    |    |    |
|            | Lb. salivarius   | 6   | 6         |     |   |   |   |    |    |    |    |    |
|            | Ped. acidilactici | 3 | 3         |     |    |   |   |    |    |    |    |
|            | W. confusa       | 5   | 5         |     |   |   |   |    |    |    |    |    |
|            | Ped. pentosaceus | 3   | 3         |     |    |   |   |    |    |    |    |    |
| ERY        | Lb. plantarum    | 10  | 1         | 7   | 2 |    |   |    |    |    |    |    |
|            | Leuc. pseudomesenteroides | 1 | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. ghanensis    | 1   | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. fermentum    | 2   | 2         |     |   |   |   |    |    |    |    |    |
|            | Lb. salivarius   | 5   | 3         | 2   |    |   |   |    |    |    |    |    |
|            | Ped. acidilactici | 3 | 2         | 1   |     |    |   |    |    |    |    |
|            | W. confusa       | 5   | 2         | 3   |    |   |   |    |    |    |    |    |
|            | Ped. pentosaceus | 3   | 2         | 1   |     |    |   |    |    |    |    |    |
| GEN        | Lb. plantarum    | 10  | 7         | 3   |    |    |   |    |    |    |    |    |
|            | Leuc. pseudomesenteroides | 1 | 1         |     |   |   |   |    |    |    |    |    |
|            | Lb. ghanensis    | 1   | 0         |     |   |   |   |    |    |    |    |    |
|            | Lb. fermentum    | 2   | 1         | 1   |    |   |   |    |    |    |    |    |
|            | Lb. salivarius   | 6   | 2         | 4   |    |   |   |    |    |    |    |    |
|            | Ped. acidilactici | 3 | 0         |     |    |   |   |    |    |    |    |
|            | W. confusa       | 5   | 4         | 1   |    |   |   |    |    |    |    |    |
|            | Ped. pentosaceus | 3   | 1         | 2   |     |    |   |    |    |    |    |    |
| KAN        | Lb. plantarum    | 10  | 0         |     |    |   |   |    |    |    |    |    |
|            | Leuc pseudomesenteroides | 1 | 0         |     |   |   |   |    |    |    |    |    |
|            | Lb. ghanensis    | 1   | 0         |     |   |   |   |    |    |    |    |    |
|            | Lb. fermentum    | 2   | 0         |     |   |   |   |    |    |    |    |    |
plantarum strains investigated in this study including strain S1 and S2 corresponded with the size of the ampli-
con obtained for the *Lb. plantarum* DSM 20174T which
was used as the reference strain and were therefore iden-
tified as such.

Similarly, unambiguous differentiation of *W. confusa*
and *W. cibaria* strains could not be achieved based on
16S rRNA gene sequencing due to the close relatedness
of the two species. However, using a species specific PCR
method reported by Fuscos et al. [39], we were able to
distinguish these two closely related species. DNA from
all the *Weissella* strains generated a PCR product with a
size of 225 bp similar to that of *W. confusa* LMG 11983T
which was used as the reference strain and no amplified
product was obtained in any of the negative control
strains (*Ped. acidilactici* DSM20284T, *Ped. pentosaceus*
DSM20314T, *Lb. paraplantarum* LTH5200, *Lb. del-
brueckii* subsp. *lactis* DSM20073, *Lb. delbrueckii* subsp.
*bulgaricus* DSM20080). The strains were therefore iden-
tified as *W. confusa*.

The reproducibility of the broth micro-dilution
method used in this study for determining the antibiotics
MIC values has been confirmed in previous studies and
is one of National Committee for Clinical Laboratory
Standards (NCCLS) recommended methods for deter-
mining antibiotic MIC values [41,46]. Our results
showed that the investigated strains were resistant to
high concentration of vancomycin. In a previous study,
Danielsen and Wind [47] shown that *Lb. plantarum/pen-
tosus* strains were resistant to higher concentrations of
vancomycin (MIC ≥ 256 μg/ml). Furthermore, *Lb. plan-
tarum, Lb. rhamnosus,* and *Lb. brevis* strains resistant to
high concentrations of vancomycin (MICs ≥ 256 μg/ml)

| Table 2 MIC distributions of 9 antibiotics for lactic acid bacteria isolated from three different African fermented food
products. Antibiotic MIC was determined by the broth microdilution method (Continued) |
|-----------------------------------------------|
| **Lb. salivarius** | 6 | 0 |
| **Ped. acidilactici** | 3 | 0 |
| **W. confusa** | 5 | 3 |
| **W. confusa** | 5 | 3 |
| **Ped. pentosaceus** | 3 | 0 |
| **STREP** | |
| **Lb. plantarum** | 10 | 2 | 5 |
| **Leuc. pseudomesenteroides** | 1 | 1 |
| **Lb. ghanensis** | 1 | 1 |
| **Lb. fermentum** | 2 | 2 |
| **Lb. salivarius** | 6 | 4 | 2 |
| **Ped. acidilactici** | 3 | 0 |
| **W. confusa** | 5 | 2 | 3 |
| **Ped. pentosaceus** | 3 | 0 |
| **TET** | |
| **Lb. plantarum** | 10 | 2 | 8 |
| **Leuc. pseudomesenteroides** | 1 | 1 |
| **Lb. ghanensis** | 1 | 1 |
| **Lb. fermentum** | 2 | 2 |
| **Lb. salivarius** | 6 | 6 |
| **Ped. acidilactici** | 3 | 1 | 2 |
| **W. confusa** | 5 | 4 | 1 |
| **Ped. pentosaceus** | 3 | 2 | 1 |
| **VAN** | |
| **Lb. plantarum** | 10 | 0 |
| **Leuc. pseudomesenteroides** | 1 | 0 |
| **Lb. ghanensis** | 1 | 0 |
| **Lb. fermentum** | 2 | 0 |
| **Lb. salivarius** | 6 | 0 |
| **Ped. acidilactici** | 3 | 0 |
| **W. confusa** | 5 | 0 |
| **Ped. pentosaceus** | 3 | 0 |

Abbreviations: AMP, Ampicillin; CHL, Chloramphenicol; CLIN, Clindamycin; ERY, Erythromycin; GEN, Gentamicin; KAN, Kanamycin; STREP, Streptomycin; TET, Tetracycline; VAN, Vancomycin. n; number of strains within each species tested. MIC range tested indicated in gray.
cin, vancomycin and streptomycin for some of the strains were higher than the recommended FEEDAP Panel's breakpoint values and were therefore considered resistant to these antibiotics and may require further molecular investigation to ascertain the cause of these resistance patterns.

Microbial strains with β-haemolytic activity unlike α-haemolytic activity produce exotoxin such as streptolysin S (SLS) which lysis blood cells and thereby affects the immune system. On blood agar plates, the blood lysis results in clearing around colonies. The general presence of poor haemolytic activities among LAB is an indication of their safety properties and is among other characteristics that accorded LAB the GRAS status. As was also observed in this study, there was generally low presence of haemolytic activity or production of streptolysin among the bacteria investigated. Only 9 out of 33 strains exhibited α-haemolytic activity and no strains showed β-haemolytic activity. It was reported by Hussain et al. [51] that out of a total of 535 enterococcal isolates, only 18 strains demonstrated haemolysis on blood agar of which 12 showed β-haemolysis and the remaining 6 strains showed α-haemolysis. Ulymaz et al. [52] also reported that Ped. pentosaceus BH105 isolated from human faeces showed no haemolytic activity on blood agar. In this study, the absence of β-haemolysis in any of the strains is a good indication of low prevalence of pathogenicity among the isolates.

Conclusions
A total of 33 LAB from three different indigenous African food products were characterised by genotypic techniques. The molecular techniques used in this study have proved successful in the identifications of the strains to species and subspecies level. The identity of some of the isolates such as Lb. fermentum ZN7b-2, ZN7b-7, Weissella confusa strains and Lb. plantarum S1 and S2 were re-established and the identity of the remaining strains confirmed.

The isolates were susceptible to ampicillin, chloramphenicol, clindamycin, tetracycline and erythromycin (except Pediococcus) and had MIC values not above the respective recommended breakpoint values for the individual species by the Panel on Additives and Products or substances used in Animal Feed (FEEDAP) [22]. However, the MIC values obtained for gentamicin, kanamycin, vancomycin and streptomycin for some of the strains were higher than the recommended FEEDAP Panel's breakpoint values and were therefore considered resistant to these antibiotics and may require further molecular investigation to ascertain the cause of these resistance patterns.
spp. to kanamycin and vancomycin indicate the prevalence of this intrinsic property among *Lactobacillus* spp. globally and thus strains of African origin do not possess any higher risk in terms of their antibiotic resistance profiles and haemolytic activities as compared to isolates of other geographical areas. Thus, the use of strains from African fermented food could be interesting as candidates of new future commercial starter cultures for selected product groups or probiotics.

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**Authors’ contribution**

DBA participated in project conception and carried out most of the experiments, analysed and interpreted the data and wrote the manuscript. DSN and LJ designed and supervised the analysis and results interpretation on molecular characterization and corrected the manuscript. KIS and PMFMD conceived the study participated in the design and supervised the work on antibiotics susceptibility profiles and haemolytic activity. All authors read and approved the final version of the manuscript.

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