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# MALDI-TOF MS profiling and exopolysaccharide production properties of lactic acid bacteria from *Kunu-zaki*-a cereal-based Nigerian fermented beverage

Omotade Richard Ogunremi [a,b], Susette Freimüller Leischtfeld [a], Susanne Miescher Schwenninger [a*]

[a] Food Biotechnology Research Group, Institute of Food and Beverage Innovation, Zurich University of Applied Sciences (ZHAW), Einsiedlerstrasse 31, 8820 Wädenswil, Switzerland

[b] Department of Biological Sciences, First Technical University, Ibadan, Nigeria

[*] Corresponding author

**Abstract**

MALDI-TOF MS is a technique for high-throughput characterization of foodborne microbiota, however, its application for studying African traditional fermented foods is limited. A total of 164 out of 220 lactic acid bacterial (LAB) isolates from *Kunu-zaki* were identified using MALDI-TOF MS, with 100% identity of representative strains compared to 16S rRNA gene sequencing. MALDI-TOF MS profiling combined with 16S rRNA gene sequencing revealed a total of 15 LAB species in *Kunu-zaki,* where the most predominant species were *Lactiplantibacillus plantarum* (40.46%), *Weissella confusa* (27.27%), and *Pediococcus pentosaceus* (15.00%). Phenotypic screening of all isolates revealed strains of *W. confusa* (57), *Lactiplantibacillus* sp. (9), *Companilactobacillus musae* (1), *Ligilactobacillus saerimneri* (1) and *Leuconostoc citreum* (1) that are capable of producing dextran and/or fructan. Dextransucrase genes were detected in all EPS-producing strains by PCR. *Weissella confusa* YKDIA1 and YKDIA4 produced 11.93 and 11.70 g/L dextran from millet-sorghum flour hydrolysate-sucrose, respectively. *Kunu-zaki* produced using *W. confusa* YKDIA1 had high water holding capacity (100%) and viscosity ranging from 49.46 – 139.24 mPas. In this study, MALDI-TOF MS adequately revealed the LAB species composition in *Kunu-zaki* in a high-throughput strategy and further, the dominant occurrence of EPS-producing LAB strains and their potentials to influence the rheological properties of *Kunu-zaki* were demonstrated.

**Keywords** Lactic acid bacteria, MALDI TOF Mass spectrometry, Cereal-based fermentation, Exopolysaccharide

## 1. Introduction

Traditional fermented cereal foods constitute a significant portion of diets in developing countries. *Kunu-zaki* is a traditional non-alcoholic fermented cereal beverage that is consumed by nearly 150 million people in Nigeria (Oguntoyinbo *et al.,* 2011). It is characterized by sweet-sour taste, creamy to reddish color and high content of suspended solids. *Kunu-zaki* is consumed as a chilled refreshing drink, with popular variants such as *Borde* in Ethiopia, *Gowe* in Benin*, Obushera* in Uganda, and *Oshikundu* in Namibia (Adinsi *et al.,* 2017; Misihairabgwi *et al.,* 2018; Mukisa *et al.,* 2012). Scientific and empirical evidences are accumulating on whole-grain and probiotic benefits of these beverages (Taylor *et al.,* 2014).

*Kunu-zaki* has ceased to be exclusively for the poor and rural dwellers but now favored by urban consumers (Oguntoyinbo *et al.,* 2011). Notwithstanding the significant improvement in the process hygiene, *Kunu-zaki* does not meet consumer demand for consistency in quality, appealing mouth-feel and reduced syneresis (Ogunremi *et al.,* 2017). Systematic selection and use of autochthonous microbial strains with inherent functional properties have been used to eliminate these defects in some fermented foods (Mukisa *et al.,* 2017). Exopolysaccharides (EPS) produced by several species of lactic acid bacteria (LAB) act as viscosifying and emulsifying agents to improve mouth-feel and reduce syneresis in fermented beverages (Zannini *et al.,* 2018).

LAB, the most predominant microorganisms in *Kunu-zaki* fermentation, largely determine the characteristics of the beverage (Ezekiel *et al.,* 2019). *Kunu-zaki* is an unexplored reservoir of LAB strains with potentials to demonstrate functional EPS production. However, detailed

identification and characterization of the LAB microbiota constitutes the basis for such selection (Pérez-Cataluña *et al.,* 2018). The golden standard and most developed molecular technique for bacterial identification remains characterization that is dedicated to sequencing of 16S rRNA genes and other sufficiently conserved species-specific genes (Nacef *et al.,* 2017; Sandrin *et al.,* 2013). This technique offers a precise identification of bacterial species. However, it is expensive and complex, requiring materials and time for sample preparation and data acquisition, analysis, and interpretation (Miescher Schwenninger *et al.,* 2016).

Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) is gaining attention as a culture-dependent technique of choice for cost-effective, accurate, and high-throughput characterization of microbiota in food ecosystems, particularly LAB in fermented foods. The method is based on the uniqueness of mass spectral patterns of ribosomal proteins of microorganisms belonging to different genera, species, and strains (Doan *et al.,* 2012). MALDI-TOF MS has previously been applied to e.g. analyze microbial ecosystem of cocoa bean fermentation (Miescher Schwenninger *et al*., 2016), and spontaneously fermenting curly kale (Michalak *et al.,* 2018), as well as LAB species composition in French Maroilles cheese (Nacef *et al.,* 2017) with comparable results to that of 16S rRNA gene sequencing. However, to the best of our knowledge, no study has reported the microbial diversity, particularly LAB species composition, of traditional fermented African foods by MALDI-TOF MS. The aim of this study was to determine the diversity of LAB isolated from *Kunu-zaki,* using MALDI-TOF MS, and check the reliability of the identification method in comparison to identification by 16S rRNA gene sequencing. Furthermore, we assayed for EPS production potentials in some isolated LAB with the aim of enhancing the functional property in *Kunu-zaki* and related products.

## 2. Materials and Methods

### 2.1. *Kunu-zaki processing*

Traditional processing of *Kunu-zaki* is presented in Fig. 1. It is prepared by steeping millet and sorghum (2:1) overnight, followed by wet-milling after addition of spice(s). The slurry obtained is divided into two unequal portions. The larger portion (2/3) is gelatinized with hot water (100 °C) and allowed to cool while the smaller portion (1/3) is mixed with ambient water. The smaller portion (1/3) and the larger gelatinized portion are mixed vigorously, then left overnight at ambient temperature for spontaneous fermentation. This is followed by filtration and sweetening to taste. The product obtained is referred to as *Kunu-zaki* (Ezekiel *et al.,* 2019).

### 2.2 *Sample collection*

A total of 13 freshly prepared *Kunu-zaki* samples was collected from local producers from June to September 2018 in three different locations in Nigeria, including Umuahia, Ibadan and Ilorin. They were transported at 4 °C to the laboratory for microbiological analysis within 24 h.

### 2.3 *LAB enumeration and isolation from Kunu-zaki*

Each sample (5 mL) was serially diluted to ten folds in 45 mL sterile saline solution. Five different agar media previously reported to isolate LAB from cereal products were used for isolation, including MRS (Man, Rogosa and Sharpe) agar containing glucose (MRS-G), maltose (MRS-M), and starch (MRS-S) at 0.5% (w/v) (Pérez-Cataluña *et al.,* 2018), MRS-5 containing maltose, fructose, and glucose at 1.0, 0.5, and 0.5% respectively (Meroth *et al.,* 2003), and Yeast Glucose Lactose Peptone agar (YGLP) (http://www.cect.org). The plates were incubated

anaerobically at 30 °C for 48 h and counts were expressed in colony forming units (CFU/mL). Morphologically distinct colonies were randomly picked and purified by successive sub-culturing on MRS agar. Gram positive and catalase negative isolates were preserved as presumptive LAB in MRS broth supplemented with 20% (v/v) glycerol at -80 °C.

### 2.4 *LAB isolates identification by MALDI-TOF MS*

Cells from fresh cultures of LAB isolates were subjected to protein extraction and treatments in MALDI-TOF MS as previously described by Miescher Schwenninger *et al.* (2016). Measurements were carried out with the Reflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) and data were analyzed with Bruker Daltonics MALDI BioTyper Software (ver. 2.0, 4110 inputs). Log scores within the range of 2.300 - 3.000 indicate highly probable species-level identification, 2.000 - 2.299 indicate highly probable genus-level and probable species-level identification, 1.700 - 1.999 indicate probable genus-level identification and 1.700 - 0.000 indicate no reliable identification.

### 2.5 *LAB isolates identification by 16S rRNA gene sequence analysis*

Amplicons of 16S rRNA gene of each LAB isolate was obtained by colony PCR as previously described by Dasen *et al.,* (1998) and purified using DNA Clean and Concentrator kit (Zymo Research, California, USA). Purified amplicons were submitted for Sanger sequencing (Microsynth AG) and compared against sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov). Sequences with more than 98% similarity were considered to belong to the same species.

2.6 *LAB isolates screening for EPS production*

LAB isolates were screened on different modified MRS agar, containing 2% (w/v) of the respective sugar, including MRS-glucose, MRS-sucrose, and MRS-raffinose (Juvonen *et al.,* 2015) and millet-sorghum flour hydrolysate-sucrose agar (MSFH-suc) that simulated the conditions in *Kunu-zaki.* For the preparation of MSFH-suc agar medium, millet-sorghum flour (2:1) in tap water (20% w/v) was incubated at 50 °C and 100 rpm for 4 h. The supernatant obtained was supplemented with 5 % sucrose, 1 % yeast extract, and 1.5 % agar (Le Lay *et al.*, 2016). All media were sterilized at 121 °C for 15 min. A 5 µL fresh MRS broth culture (30 $^{o}$C/24 h) of each isolate was spotted on the surface of sterile MRS-glucose, MRS-sucrose, MRS-raffinose and MSFH-suc agars, and incubated anaerobically at 30 $^{o}$C for 48 h. Colonies were assessed for slime mass and length. EPS production was scored as "-", "+" , "++", and "+++" representing slime length of "0 mm", "< 1.5 mm", "1.5-3 mm", and ">3 mm", respectively. The average score of duplicate measurements was calculated.

2.7 *Determination of EPS coding genes in EPS-producing LAB isolates*

The presence of genes that code for proteins required for EPS biosynthesis, including *dex* (dextransucrase) and *ftf* (fructansucrase), were determined in EPS-producing LAB isolates using primers and PCR conditions presented in Table 1. Strains that did not produce EPS were included as a negative control.

2.8 *Isolation, quantification and characterization of EPS*

For the isolation of EPS, selected EPS-producing LAB strains (1% v/v) were grown (30°C/24h) in 1000 mL MSFH-suc. EPS were isolated as described by Abid *et al.* (2018) with slight modification. Following dialysis with 10 kDa molecular weight cut-off, dialyzed EPS solution was frozen at -20 $^{O}$C and lyophilized at -50 $^{O}$C for 48 h and the dry weight of EPS was determined. The neutral carbohydrate content, being an estimation of EPS concentration, was determined by phenol-sulfuric acid method using glucose as the standard (Dubois *et al.,* 1956) while the protein content was determined by Folin-Coicalteau method (Lowry *et al.,* 1951). The monosaccharide composition of EPS was analyzed after hydrolysis by HPLC analysis as reported by Dertli *et al.* (2018). The freeze-dried EPS was hydrolysed with 0.5 M $H_2SO4$ at 95 °C for 6 h followed by neutralisation (pH 7) with 4 M NaOH. The hydrolysates were filtered through a 0.2 µm pore size filter and the monosaccharide composition was determined by HPLC. The HPLC machine (Agilent Technologies 1260 Infinity) was equipped with Rezex$^{TM}$ RPM-Monosaccharide Pb+2 column (300 X 7.8 mm) and refractive index detector (RID, G1362A, Agilent Technologies). The mobile phase was Millipore water with a flow rate of 0.6 ml/min. Glucose and fructose were used as standard monosaccharides.

2.9. *Kunu-zaki preparation by controlled fermentation with EPS-producing LAB strain*

Controlled fermentation of millet-sorghum slurry, using starter-culture of *Lactiplantibacillus plantarum* strain SKMIM1 (high acid-producing strain belonging to the most predominant species in *Kunu zaki*) and/or high EPS-producing *Weissella confusa* YKDIA1 to prepare *Kunu-zaki* is described in Fig. 2. Fresh culture (30$^{o}$C/12h) of inoculum was enumerated (1X10$^{9}$ CFU/ml) and washed twice in equal volume of sterile saline solution (0.89% NaCl). Mixed strains starter of *L.*

*plantarum* SKMIM1 and *W. confusa* YKDIA1 (1:1) and single strain starter of *W. confusa* YKDIA1 were inoculated (1% v/v) into separate millet-sorghum slurry models, to start the fermentation process at 30 °C for 16 h. An uninoculated millet-sorghum slurry model was used as negative control. Samples were taken before and after fermentation for LAB enumeration on MRS agar plates and pH determination using a pH meter (Knick pH-meter 766 Calimatic, Berlin, Germany). A 1-mL portion of each sample was used to determine glucose, sucrose, fructose, maltose, lactic acid and acetic acid contents in a HPLC system (Agilent Technologies 1260 Infinity) (Romanens *et al.,* 2019). Viscosity and water holding capacity (WHC) were determined in *Kunu-zaki* that were supplemented with different concentrations (0, 1.5 and 5%) of sucrose before fermentation. Viscosity measurement in 50 mL of samples was made using HAAKE ViscoTester VT550 (Thermo Scientific, Germany) equipped with sensory rotor (MV DIN 53019/ ISO 3219) and measuring cup (MV DIN). Viscosity values were taken after samples were sheared at the rate of 200/s for 2 mins at 25$^{o}$C. WHC was determined as described by Zannini *et al.* (2018).

### 2.10 *Statistical analysis*

Results were presented as means with standard deviation of replicate values after they were subjected to one-way analysis of variance (ANOVA) and compared for significant differences using Tukey's HSD test. Values of $p < 0.05$ were considered to be statistically significant.

## 3. Results

### 3.1 *LAB enumeration and isolation from Kunu-zaki*

All 13 samples of *Kunu-zaki* analyzed in this study revealed presumptive LAB on all agar media, where LAB colonies appeared typically smooth, round with opalescent, or translucent color. Viable counts of LAB were within the range of 8 – 10 Log CFU/mL. A total of 220 isolates were randomly recovered from the five different isolation media, including 45, 50, 45, 31, and 49 isolates from MRS-G, MRS-M, MRS-5, MRS-S, and YGLP, respectively. The purified colonies were considered as presumptive LAB due to their Gram positive and catalase negative biochemical characteristics.

3.2 *LAB biodiversity of Kunu-zaki by MALDI-TOF MS and 16S rRNA gene sequencing*

Analysis of the 220 LAB isolates by MALDI-TOF MS provided BioTyper scores within the range of 1.70 - 2.61, identifying 164 isolates (74.55%) and 56 (25.45%) with scores below 1.70. Among the identified isolates, 64 (29.09 %), 58 (26.36%), and 42 isolates (19.09 %) had BioTyper scores of 2.300- 3.000, 2.000- 2.299, and 1.700– 1.999, respectively (Table 2). MALDI-TOF MS identified the isolates as species of *Enterococcus, Companilactobacillus, Lactiplantibacillus, Lactobacillus, Lactococcus, Latilactobacillus, Levilactobacillus, Leuconostoc, Ligilactobacillus, Limosilactobacillus, Pediococcus,* and *Weissella*. (Table 3).

Among the predominant species, two strains each of *L. plantarum, W. confusa*, and *Pediococcus pentosaceus* and one strain each of *Limosilactobacillus fermentum* and *Lactococcus lactis* revealed a 100% match in their species identities using 16S rRNA sequencing and MALDI TOF MS (Table 3). Further, 16S rRNA gene sequence analyses of the 56 unidentified strains based on MALDI-TOF MS identification revealed *W. confusa* (37 strains), *P. pentosaceus* (10 strains), *W. paramesenteroides* (five strains), *Companilactobacillus musae* (two strains), *Lactobacillus*

*delbrueckii* (one strain), and *L. plantarum* (one strain) (Table 3). The 16S rRNA gene sequences of the selected LAB isolates are available in the NCBI nucleotide sequence database under accession numbers HG970178 to HG970189 (http://www.ncbi.nlm.nih.gov/Genbank/).

Combining data of MALDI-TOF MS and 16S rRNA gene sequencing of all 220 LAB isolates, revealed 15 species with predominance and widespread occurrence of *L. plantarum* (40.46 %), *W. confusa* (27.28 %), and *P. pentosaceus* (15.00 %) in *Kunu-zaki.* Other significant species include *L. lactis, W. paramesenteroides, L. fermentum, L. delbrueckii, C. musae* and *L. pentosus.* (Table 3).

3.3 *EPS production potential by LAB isolates*

As evidenced on MRS-suc and MSFH agar plates (Fig. 3), 69 LAB strains belonging to, *C. musae, L. plantarum, L. pentosus, L. saerimneri, Leuc. Citreum*, and *W. confusa* displayed different masses of mucoid and slime lengths on MRS-suc and MSFH-suc agar plates, indicating EPS production, particularly dextran and/or fructan (Fig. 4). EPS production was not observed on MRS-glu that would indicate the production of capsular polysaccharide, while a single strain; *Leuc. Citreum* 5KDIA7 produced EPS on MRS-raf agar, suggesting fructan production (Table 4).

3.4 *Detection of genes responsible for EPS production*

The use of primer pair WConDex fw/WConDex rev revealed that all the test *W. confusa* strains and *L. saerimneri* GKJEU6 harbored the gene coding for *W. confusa* dextransucrase (1037 bp size), which catalyses dextran production (Table 4). However, primer pair Dexreu V/ Dexreu R generated amplicons of dextransucrase gene (600 bp size) in only 11 of the 17 EPS-positive LAB

strains (Fig. 5). Dextransucrase gene was not detected in *Leuc. citreum* 5KDIA7 using both primer pairs. Additionally, no amplicons were obtained for all isolates with primer FTF 2-F targeting fructansucrase gene (Table 4).

3.5 *Isolation, quantification and characterization of EPS*

The cell-free supernatants of the respective MSFH-suc broth culture of *W. confusa* YKDIA1 and *W. confusa* YKDIA4 yielded 11.93 and 11.70 g/L of EPS dry weight (Table 5). The total sugar and protein contents of both EPSs were more than 90% and approximately 1% respectively (Table 5). Acid hydrolysis and HPLC analysis of EPSs for monosaccharide composition revealed a single peak, with retention time that corresponded to glucose (Figure S1 and S2). The EPSs from both strains are composed of only glucose as analyzed by HPLC (Table 5).

3.6 *Microbiological and physicochemical properties of Kunu-zaki*

Millet-sorghum slurry supported the growth of the LAB strains with cell density increases by 1.0-1.2 log units within 16 h of fermentation (Table 6). The fermentations resulted in rapid acidification of the slurries, decreasing their pHs from 6.27 to 3.60 and 3.40 when fermented with *W. confusa* YKDIA1 and *L. plantarum* SKMIM1 and *W. confusa* YKDIA1, respectively (Table 6).

After fermentation, sucrose was completely metabolized from 46.82 g/L and fructose accumulated from 4.52 g/L up to 21.50-21.89 g/L in both test samples (Table 6). Higher concentration of lactic acid (5.64 g/L) was produced in *Kunu-zaki* sample started with *L.*

*plantarum* SKMIM1 and *W. confusa* YKDIA1, while higher concentration of acetic acid (0.21 g/L) was produced in the sample started with *W. confusa* YKDIA1 (Table 6).

3.7 *Rheological properties of Kunu-zaki*

The viscosity of sucrose-free inoculated samples decreased after fermentation to 26.12-26.56 mPas, compared to control samples with viscosity of 29.48 mPas. Addition of sucrose (1.5% and 5% w/v) resulted to significant increases ($p< 0.05$) in viscosity of samples that were fermented with *W. confusa* YKDIA1 and *L. plantarum* SKMIM1 and *W. confusa* YKDIA1, indicating the proportionate production of EPS and the corresponding thickening effects. The highest viscosities were found in *Kunu-zaki* samples that were supplemented with 5% sucrose and fermented with *W. confusa* YKDIA1 (139.24 mPa.s). Viscosities of *Kunu-zaki* samples enriched with 1.5% sucrose ranged from 49.46 to 56.39 mPa.s (Table 7). Among the *Kunu-zaki* samples, all sucrose-free and control samples showed relatively lower WHC. The WHC of starter-produced *Kunu-zaki* samples were significantly higher ($p < 0.05$) due to EPS produced by *W. confusa* YKDIA1 (Table 7)

## 4. Discussion

Lactic acid bacteria (LAB) are the predominant group of microorganisms involved in the spontaneous fermentation of cereal food substrates (Banwo *et al.,* 2020). The high counts of LAB (Log 8-10) in *Kunu-zaki* samples reported in this study affirms previous reports on African traditional fermented cereal foods (Adepehin *et al.,* 2018). Few studies based on molecular techniques to reveal the complex species and strain diversity of the LAB community in

traditional fermented foods of Africa principally used PCR-based gene analysis (Ezekiel *et al.,* 2019; Oguntoyinbo *et al.,* 2011).

This study reports, for the first time, the application and validation of MALDI-TOF MS to identify LAB species in *Kunu-zaki*, a traditional fermented food of Nigeria. In contrast to the PCR-based gene approaches, MALD-TOF MS profiling has proven to be a cost-effective, and high-throughput technique for characterization of microbiota in food ecosystems (Doan *et al.,* 2012; Miescher Schwenninger et al., 2016). In this study, 74.55 % of the 220 LAB isolates were identified, resulting in 15 species. This identified proportion is lower compared to reports of Duskova *et al.* (2012) and Michalak *et al.* (2018) where 98.0 % and 97.73 % of LAB isolates from traditional fermented foods of European origin were respectively identified by MALDI-TOF MS. Similar to our study, high proportion of unidentified food associated microbial strains were documented for spontaneously fermented cocoa bean and breast skin of chicken carcasses (Miescher Schwenninger *et al.,* 2016; Yu *et al.,* 2019). The limited successes achieved in profiling food microbiota with MALDI-TOF MS was suggested to be due to insufficient number of strains in the Biotyper database to compensate for the intraspecies diversity (Gantzias *et al.,* 2020). This is prominent for specific species from exotic food sources (Michalak *et al.,*2018; Miescher Schwenninger *et al.* 2016) and was confirmed when the expansion of the Biotyper database allowed the successful identification of cocoa-specific acetic acid bacteria (Miescher Schwenninger *et al.* 2016) and *Weissella* species (Lee *et al.,* 2015).

In this study, the suitability of MALDI-TOF MS to reveal the species composition of LAB in *Kunu-zaki* was established by the comparable identification using 16S rRNA gene sequence analyses.

Similarly, previous studies on LAB species composition of food products showed consistency between results of MALDI-TOF MS and 16S rRNA gene sequence analysis (Doan *et al.,* 2012; Duskova *et al.,* 2012; Gantzias *et al.,* 2020; Nacef *et al.,* 2017). The high proportions of unidentified strains of *W. confusa* (37/60)*, W. paramesenteroides* (5/7), and *P. pentasaceus* (10/33) by MALDI-TOF MS in this study could be due to the limited number of reference strains of *W. confusa, W. paramesenteroides*, and *P. pentasaceus* in the Biotyper database and the exotic nature of the food from where the strains were isolated. Lee *et al.* (2015) noted that the current reference spectra were not sufficient to identify *W. confusa*.

Overall, MALDI-TOF MS and 16S RNA successfully revealed the predominance of *L. plantarum, W. confusa*, and *P. pentosaceus* in *Kunu-zaki* samples. Other important species included *Lactococcus lactis, L. fermentum, L. delbrueckii*, and *W. paramesenteroides.* These LAB species are suggested to play vital roles during *Kunu-zaki* processing and they constitute potential strains for use as starter cultures. Similar patterns of distribution have been reported in previous studies, where, Ezekiel *et al.* (2019) and Adepehin *et al.* (2018) respectively reported the dominance of *Lactobacillus, Pediococcus*, and *Weissella* in *Kunu-zaki* and gluten-free sourdough based on pearl millet and sorghum. Additionally, similar species of LAB were reported in fermented millet beverages of Burkina faso, *i.e. Ben-saalga* (Turpin *et al.,* 2011) and Uganda, *i.e. Obushera* (Mukisa *et al.,* 2012).

This study reports for the first time the occurrence of *C. musae* and *L. saerimneri* in a cereal-based traditional fermented food of Nigeria that could be explained by the wider range of isolation media used. In this study, *C. musae* strains were isolated on MRS-maltose and MRS-

starch agar. In addition, process variations, choice of additives, and geographical origin of the samples of this study could have influenced the occurrence of unfamiliar species. *Companilactobacillus musae* was first isolated from banana fruit (*Musa paradisiaca* var. *sapientum*) in Taiwan and named after the fruit source (Chen *et al.,* 2017).

The potential to produce exopolysaccharide is an interesting property of LAB for application in fermented beverages as natural viscosifying and emulsifying agents (Jeske *et al.,* 2018; Zannini *et al.,* 2018). The dominant occurrence of EPS-producing LAB strains in *Kunu-zaki* from this study was also reported for LAB isolates from soya and pureed carrot (Juvonen *et al.,* 2015; Malik *et al.,* 2009). Production of EPS was mainly distributed among strains of *Lactiplantibacillus* sp., *W. confusa*, and *Leuc. citreum.* Considering, their predominance in plant sources, these species mainly produce homopolysaccharide EPS, including glucans and fructans (Dertli *et al.,* 2016; Juvonen *et al.,* 2015). A widespread distribution of glucan production among LAB species from *Kunu-zaki* was observed, while frutan-like EPS were only noted for *Leuc. citreum* 5KDIA7. As in this study, Malang *et al*. (2015) described *W. confusa* as prominent glucan producer. Furthermore, the production of EPS on MSFH-suc agar, and thus of homopolysaccharides, is an indication of the feasibility and suitability of the producing strains to be applied in fermentation process for the affordable and sustainable improvement of rheological properties of *Kunu-zaki.* In this study, the detection of dextransucrase genes in 11 and 17 LAB strains using primer pairs WConDex fw/WConDex rev (Malang *et al.,* 2015) and Dexreu V/ Dexreu R (Tieking *et al.,* 2003) respectively further confirms the production of glucan by EPS-producing LAB strains. The failure to obtain amplicons of fructansucrase gene with the primer pair FTF 2-F/FTF 2-R (Bounaix *et al.,* 2009) for all test LAB strains, including *Leuc. citreum* 5KDIA7, which phenotypically produced

fructan may confirm inability to produce fructan or species limitation of primer pair (Tieking *et al.,* 2005).

The abundant EPS yield of 11.93 and 11.70 g/L by *W. confusa* YKDIA1 and YKDIA4 in MSFH-suc (5% sucrose) is an indication of the significant impact that the producing strain can have on improved mouthfeel and reduced syneresis in *Kunu-zaki.* Considering the relatively higher yield, these strains may be interesting as potential sources of EPS additive for application in other food milieus. Some previous reports gave lower yields of 5.10 g/L by *W. confusa* AJ53 (Dubey and Jeevaratnam, 2015) and 3.00 g/L by *W. confusa* OF126 (Adesulu-Dahunsi *et al.,* 2018), despite optimized conditions. However, a higher EPS yield of 17.2 g/L was reported for *W. confusa* KR780676 (Kavitake *et al.,* 2016). Glucose as the sole monosaccharide detected by HPLC after hydrolysis of both EPSs, indicate that the *W. confusa* strains produce an homopolysaccharide-dextran. This has been generally reported for EPS isolated from *W. confusa* (Malang *et al.,* 2015).

The cereals that constitute the composite and additives used for *Kunu-zaki* production contains suitable nutritional components to support LAB growth and metabolism. This was confirmed by the luxurious growth and significant reduction of pH, changes in sugar profile and accumulation of organic acids by both strain of *W. confusa* YKDIA1 and *L. plantarum* SKMIM1 during the fermentation of millet-sorghum slurry. Comparable increase in cell density of LAB and reduction in pH during fermentation of millet dough was earlier reported (Adepehin *et al.,* 2018). The lower pH in the mixed-starter fermented sample is due to the higher concentration of lactic acid produced by the homofermentative *L. plantarum* SKMIMI. The complete depletion of

sucrose reported in this study is in line with the capacity of EPS-producing LAB strains to metabolize sucrose initially for growth during exponential phase, then hydrolyzed by the activity of dextransucrase to liberate glucose and fructose, whereby glucose is used for dextran production during stationary phase (Xu *et al.,* 2017). The high concentration of fructose and low concentration of acetic acid in *W. confusa* YKDIA1 started *Kunu-zaki* samples is as a result of the inability of *W. confusa* to metabolize the liberated fructose to mannitol and acetate, due to lack of mannitol dehydrogenase (Galle *et al.,* 2010).

Increasing supplementation of sucrose and fermentation with EPS-positive strain significantly increased the viscosity and WHC of *Kunu-zaki,* compared to control and sucrose-free samples. Besides, they did not exhibit phase separation. This is attributed to water-binding capacity of EPS and interactions with cereal proteins (Xu *et al.,* 2017; Zannini *et al.,* 2018). Similarly, results were reported after sucrose enrichment of quinoa milk and fermentation with EPS-producing *W. cibaria* (Zannini *et al.,* 2018).

## 5. Conclusions

This study, for the first time, demonstrated the accuracy of MALDI-TOF MS to identify LAB isolates from a Nigerian fermented beverage, i.e., *Kunu-zaki.* However, the database should be expanded to compensate for intraspecies diversity with emphasis on LAB species and strains from exotic food sources, including indigenous fermented foods. The predominant LAB species in *Kunu-zaki* were *L. plantarum, W. confusa*, and *P. pentosaceus,* which are typical species for traditional cereal fermentations. Within the *Kunu-zaki* LAB microflora, we recognized a dominant occurrence of EPS-producing strains, as exhibited by mucoidal colonies and

dextransucrase encoding *dex* genes harbored at strain-specific levels. Concomitant with the early integration of a MSFH (pH 6.6) medium, simulating the conditions of *Kunu-zaki*, these findings could form a basis for the development of functional starter cultures to improve the rheological properties of *Kunu-zaki* and related traditional fermented beverages.

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**Table 1:** Primers and PCR conditions used to detect genes required for EPS biosynthesis in lactic acid bacteria

| Primer | Sequence (5' – 3') | Target gene | Amplicon size | PCR conditions | Reference |
|---|---|---|---|---|---|
| WConDex fw<br>WConDex rev | TGTGGATTCAGGACACCGTA<br>GGTTCAATCACGGCTAACG | *W. confusa* dextransucrase | 1037 bp | 1 X (94 °C, 4 min.); 30 X (94 °C, 30 s; 55 °C, 1 min; 72 °C, 100 s); 1 X (72 °C, 7 min) | Malang *et al.,* 2015 |
| Dexreu V<br>Dexreu R | GTGAAGGTAACTATGTTG<br>ATCCGCATTAAAGAATGG | Dextransucrase | 600 bp | 1 X (95 °C, 3 min.); 35 X (94 °C, 30 s; 55 °C, 45 s; 72 °C, 1 min); 1 X (72 °C, 6 min) | Tieking *et al.,* 2003 |
| FTF 2-F<br>FTF 2-R | GAYRTYTGGGAYWSNTGGC<br>GCWGANCCNGACCATTSTTG | Fructansucrase | 220 bp | 1 X (94 °C, 5 min.); 30 X (94 °C, 45 s; 55 °C, 30 s; 72 °C, 30 s); 1 X (72 °C, 10 min) | Bounaix *et al.,* 2009 |

**Table 2:** Percentage proportions of identification scores of 220 LAB isolates from *Kunu-zaki* by MALDI-TOF MS

| S/N | MALDI-TOF MS Log Scores range | Interpretation of scores | Number of isolates | Percentage proportion (%) |
|---|---|---|---|---|
| 1 | 2.300 - 3.000 | Highly probable species identification | 64 | 29.09 |
| 2 | 2.000 - 2.299 | Secure genus identification and probable species identification | 58 | 26.36 |
| 3 | 1.999 - 1.700 | Probable genus identification | 42 | 19.09 |
| 4 | 1.699 - 0.000 | Not reliable identification | 56 | 25.45 |

**Table 3:** Lactic acid bacterial species composition of *Kunu-zaki* as revealed by MALDI-TOF MS combined with 16S rRNA sequencing

| Species | Percentage (No of isolates) | No of strains identified | | Comparison of identification of representative LAB species | | |
|---|---|---|---|---|---|---|
| | | MALDI-TOF MS | 16S rRNA gene sequencing [a] | Representative strain [b] | MALDI-TOF MS Score | 16S rRNA gene sequence % similarity (Accession number [c]) |
| *E. faecium* | 0.46 (1) | 1 | - | - | - | - |
| *C. musae* | 1.36 (3) | 1 | 2 | - | - | - |
| *L. paraplantarum* | 0.91 (2) | 2 | - | - | - | - |
| *L. plantarum* | 40.46 (89) | 88 | 1 | SKMIM4 | 2.53 | 99 (MN104795) |
| | | | | YKDIA2 | 2.61 | 99 (MN104756) |
| *L. pentosus* | 1.36 (3) | 3 | - | - | - | - |
| *L. delbrueckii* | 2.27 (5) | 4 | 1 | - | - | - |
| *Lc. lactis* | 3.18 (7) | 7 | - | MKDIA2 | 2.28 | 99 (MN104742) |
| *L. curvatus* | 0.46 (1) | | - | - | - | - |
| *L. brevis* | 0.46 (1) | 1 | - | - | - | - |
| *Leuc. citreum* | 0.46 (1) | 1 | - | - | - | - |
| *L. saerimneri* | 0.46 (1) | 1 | - | - | - | - |
| *L. fermentum* | 2.73 (6) | 6 | - | 5KJEU5 | 2.21 | 99 (MN104765) |
| *P. pentosaceus* | 15 (33) | 23 | 10 | GKMIO1 | 2.24 | 99 (MN104770) |
| | | | | MKJIO7 | 2.48 | 99 (MN104779) |
| *W. confusa* | 27.27 (60) | 23 | 37 | MKJEU2 | 2.19 | 99 (MN104762) |
| | | | | 5KDIA8 | 2.48 | 99 (MN104749) |
| *W. paramesenteroides* | 3.18 (7) | 2 | 5 | - | - | - |

[a] Strains with not reliable identification (NRI) by MALDI-TOF MS were identified by 16S rRNA sequencing

[b] All representative strains identified by MALDI-TOF MS gave comparable identification by 16S rRNA sequencing

[c] GenBank (http://www.ncbi.nlm.nih.gov/Genbank/)

**Table 4:** EPS production on different screening media and detection of homopolymeric EPS genes (*dex* and *ftf*)

| Strain | EPS production [a] | | | Dextran gene (*dex*) [b] | | Fructan gene (ftf) [b] |
|---|---|---|---|---|---|---|
| | MRS-Suc agar | MRS-Raf agar | MSFH-Sucrose agar | DexreuV DexreuR | WConDexfw WConDexrev | |
| *W. confusa* GKDIA6 | +++ | - | + | - | + | - |
| *W. confusa* GKDIA7 | +++ | - | + | - | + | - |
| *Leuc. citreum* 5KDIA7 | + | + | + | - | - | - |
| *W. confusa* YKDIA1 | +++ | - | ++ | - | + | - |
| *W. confusa* YKDIA4 | +++ | - | ++ | - | + | - |
| *W. confusa* GKJEU5 | +++ | - | + | + | + | - |
| *L. saerimneri* GKJEU6 | +++ | - | ++ | + | + | - |
| *W. confusa* MKJEU3 | +++ | - | ++ | + | + | - |
| *W. confusa* MKJEU5 | +++ | - | ++ | + | + | - |
| *W. confusa* 5KJEU8 | +++ | - | + | + | + | - |
| *W. confusa* SKJEU1 | +++ | - | ++ | + | + | - |
| *W. confusa* SKJEU7 | +++ | - | ++ | + | + | - |
| *W. confusa* YKJEU1 | +++ | - | ++ | + | + | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| *W. confusa* YKJEU4 | +++ | - | + | + | + | - |
| *W. confusa* YKJEU6 | ++ | - | + | + | + | - |
| *W. confusa* YKJEU7 | +++ | - | + | + | + | - |
| *W. confusa* MKMIN6 | +++ | - | ++ | - | + | - |
| *W. paramesenteroides* 5KRMA4 (control) | - | - | - | - | - | - |
| *W. confusa* MKJEU2 (negative control) | - | - | - | - | - | - |

[a] "-" , "+" , "++" and "+++" representing slime mass and length of "0 mm", "< 1.5 mm", "1.5-3 mm" and ">3 mm"
[b] Presence of the corresponding gene, e no detection of the corresponding gene

**Table 5.** Properties of EPS produced by *W. confusa* strains in MSFH-suc broth

| Parameter | *W. confusa* YKDIA1 | *W. confusa* YKDIA4 |
|---|---|---|
| EPS yield (g/L) | 11.93 | 11.70 |
| Sugar content of EPS (%) | 92.1 | 91.2 |
| Protein content of EPS (%) | 1.11 | 1.01 |
| Monosaccharide composition of EPS | Glucose | Glucose |

**Table 6.** Microbiological and physicochemical properties of *Kunu-zaki*

| Parameter | 0 h | 16 h | | |
|---|---|---|---|---|
| | | Control | *W. confusa* YKDIA1 | *L. plantarum* SKMIM1 + *W. confusa* YKDIA1 |
| pH | 6.27±0.00 [d] | 5.42±0.05 [c] | 3.60±0.01 [b] | 3.40±0.01 [a] |
| Sugar profile (g/L) | | | | |
| Glucose | 2.46±0.02 [a] | 5.59±0.05 [d] | 2.70±0.02 [b] | 4.11±0.02 [c] |
| Sucrose | 46.82±1.20 [c] | 28.66±1.84 [b] | ND | ND |
| Fructose | 4.52±0.07 [a] | 8.80±0.92 [b] | 21.50±0.08 [c] | 21.89±0.39 [c] |
| Maltose | ND | ND | 0.54±0.00 [b] | 0.35±0.10 [b] |
| Acid profile (g/L) | | | | |
| Lactic acid | ND | 0.41±0.18 [a] | 4.45±0.03 [b] | 5.64±0.10 [c] |
| Acetic acid | ND | ND | 0.21±0.00 [c] | 0.16±0.00 [b] |

ND: Not detectable. Values are given as means ± SD of data from replicate experiments. Values in the same row with different superscript differ significantly ($p$< 0.05)

**Table 7.** Rheological properties of *Kunu-zaki* prepared with different concentrations of sucrose

| *Kunu-zaki* samples | Viscosity (mPas)[1] | Water Holding Capacity (%) |
|---|---|---|
| 0% sucrose | | |
| Uninoculated control | 29.48±2.61 [a] | 95.14±0.10 [c] |
| *W. confusa* YKDIA1 | 26.12±2.20 [a] | 95.43±0.34 [c] |
| *L. plantarum* SKMIM1 + *W. confusa* YKDIA1 | 26.56±1.09 [a] | 94.68±0.09 [c] |
| 1.5% sucrose | | |
| Uninoculated control | 28.34±0.43 [a] | 89.83±0.98 [b] |
| *W. confusa* YKDIA1 | 56.39±2.33 [b] | 100.00±0.00 [d] |
| *L. plantarum* SKMIM1 + *W. confusa* YKDIA1 | 49.46±0.83 [b] | 100.00±0.00 [d] |
| 5% sucrose | | |
| Uninoculated control | 22.74±2.61 [a] | 87.14±1.17 [a] |
| *W. confusa* YKDIA1 | 139.24±4.55 [d] | 100.00±0.00 [d] |
| *L. plantarum* SKMIM1 + *W. confusa* YKDIA1 | 101.78±1.95 [c] | 100.00±0.00 [d] |

[1] Viscosity values of samples were taken after shearing at the rate of 200 1/s for 120 sec at 25°C

Values are given as means ± SD of data from replicate experiments. Values in the same column with different superscript differ significantly ($p< 0.05$)

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

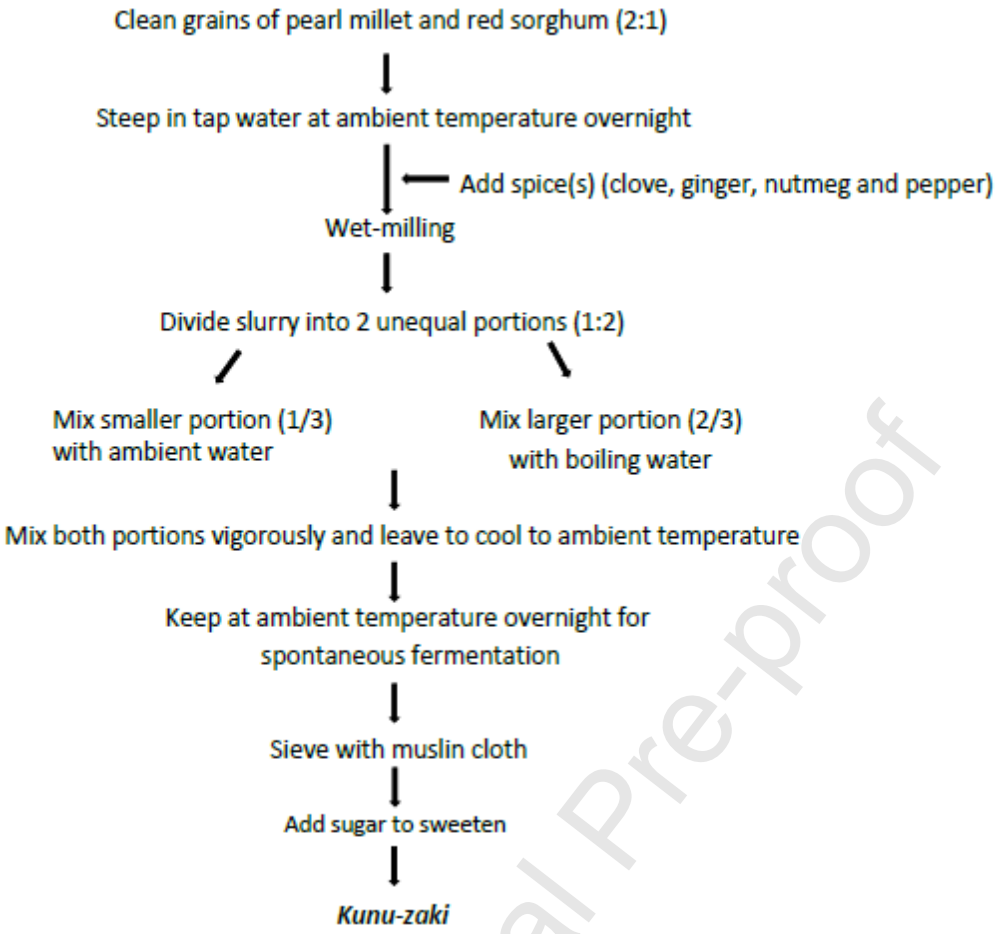


**Fig. 1.** Flow chart for traditional preparation of *Kunu-zaki*

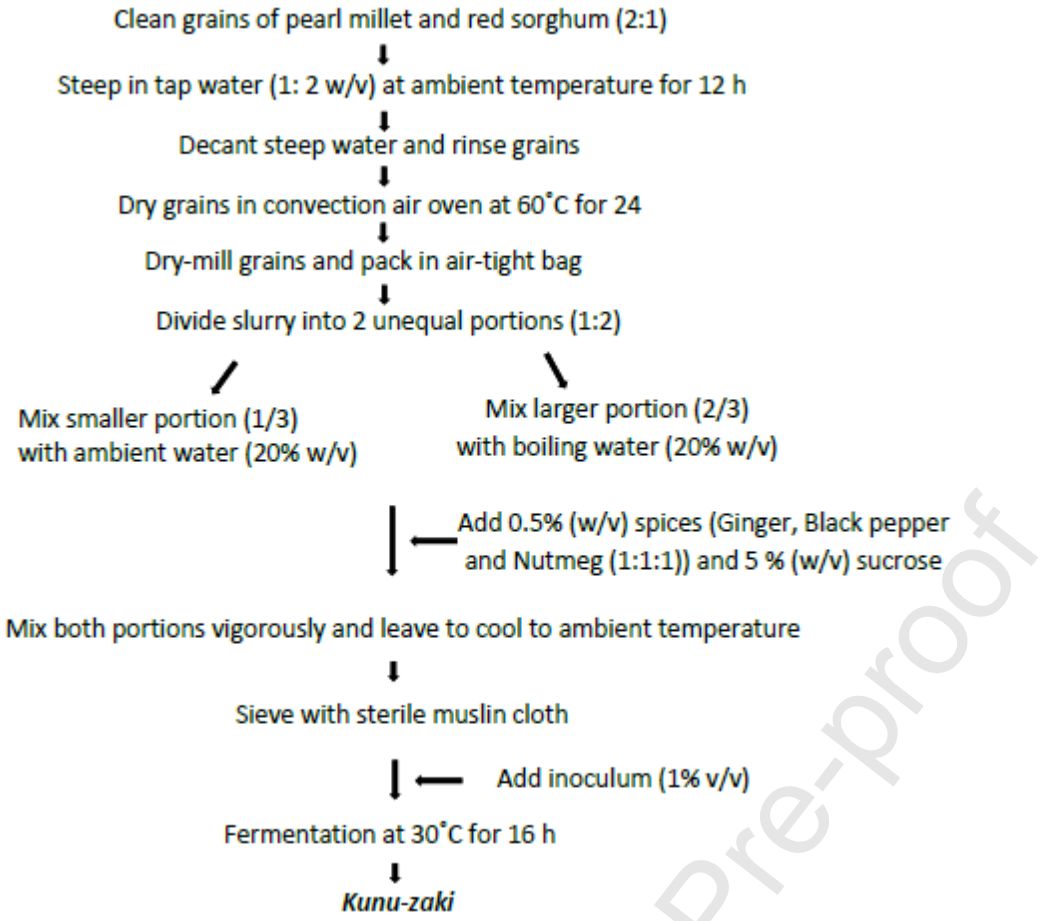


**Fig. 2.** Flow chart for laboratory preparation of *Kunu zaki* using starter-culture

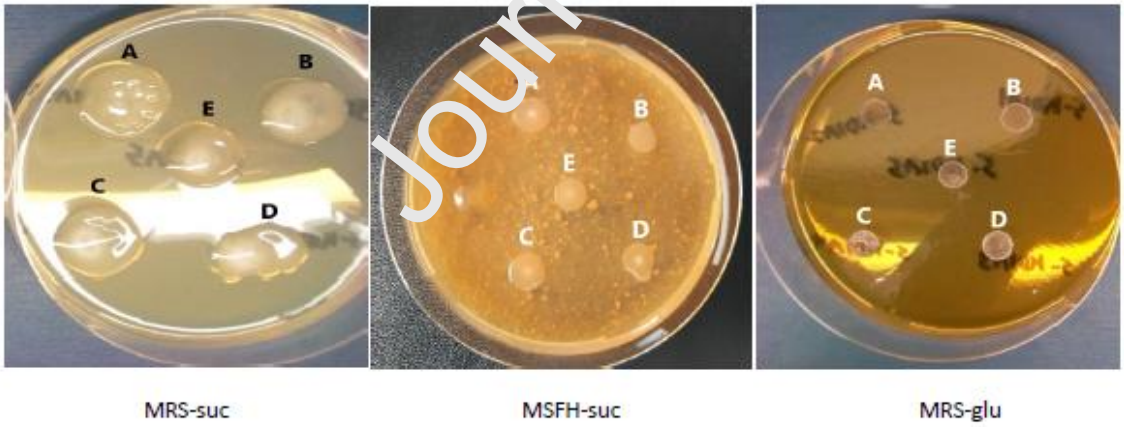


**Fig. 3.** EPS production by LAB on MRS-suc, MSFH-suc, and MRS-glu (control) agar plates after 48 h of incubation at 30 ˚C. A: *W. confusa* 5KDIA1, B: *W. confusa* 5KDIA2, C: *W. confusa* 5KDIA3, D: *W. confusa* 5KDIA4, E: *W. confusa* 5KDIA5

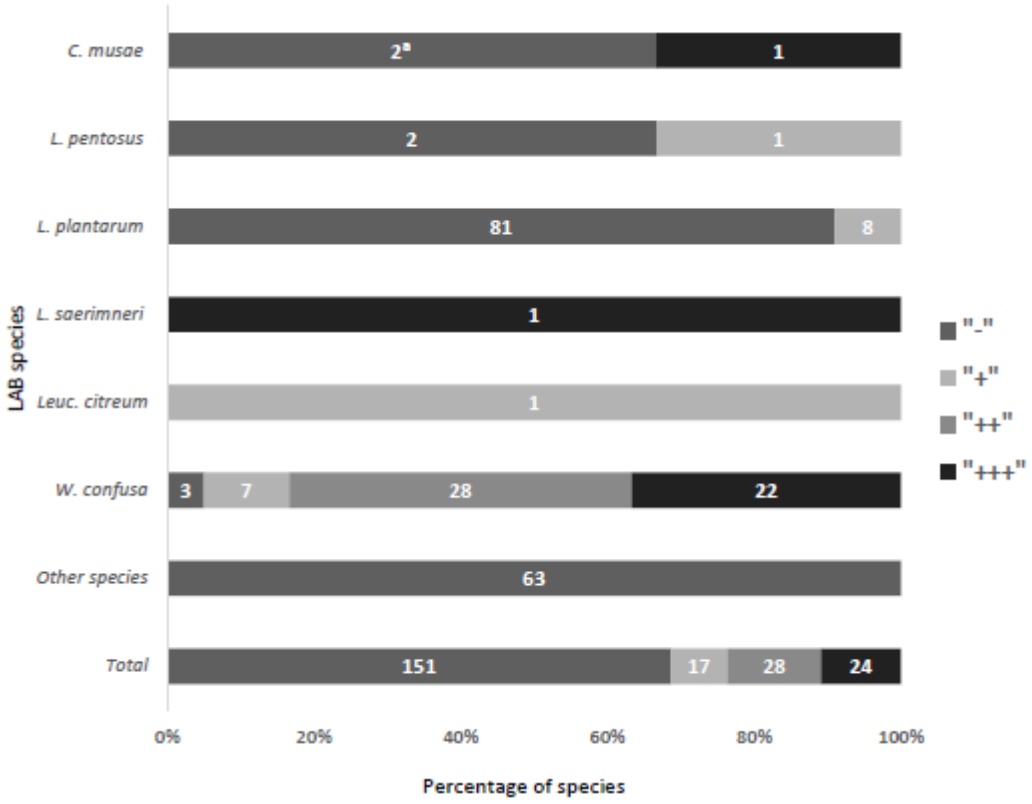


**Fig. 4:** Exopolysaccharide production on MRS-Suc agar by LAB isolates from *Kunu zaki.* Other species include: *E. faecieum, L. brevis, L. curvatus, L. delbrueckii, L. fermentum*, *L. paraplantarum, Lc. lactis, P. pentosaceus*, *W. paramesenteroides.* "a"Numbers on the bars indicate number of LAB strain of each species in each EPS production group. "-" , "+" , "++" and "+++" represent slime mass and length of "0 mm", "< 1.5 mm", "1.5-3 mm", and "3 mm" respectively.

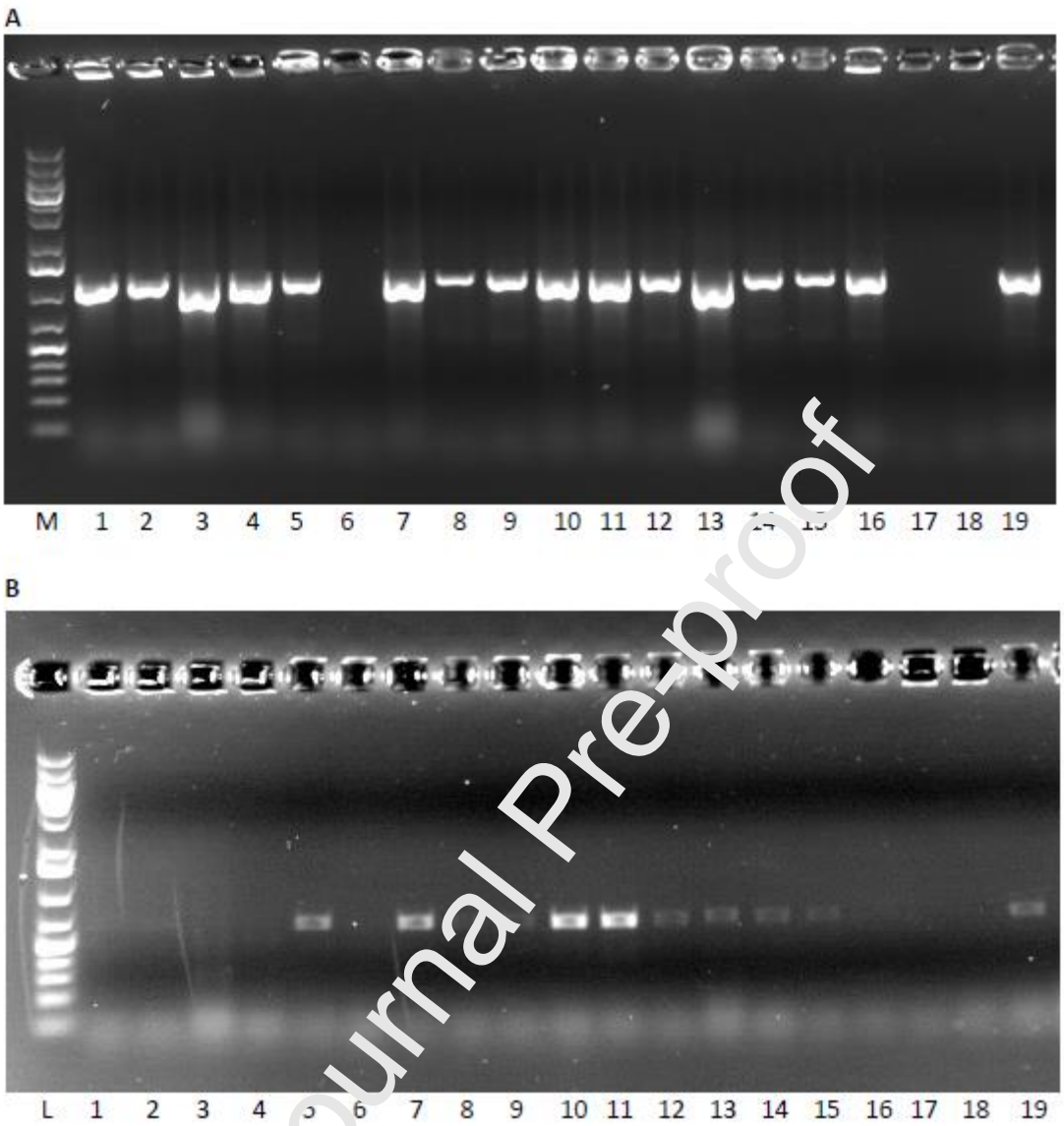




**Fig. 5.** Agarose gels (1% w/v) showing the amplicons of dextransucrase gene (dex) using primer pairs (A) WconDex fw and WconDex rev; (B) DexreuV and DexreuR. L: 1kb plus DNA ladder; 1 – 16: *W. confusa* GKDIA6, GKDIA7, YKDIA1, YKDIA4, GKJEU5, MKJEU2 (control), MKJEU3, MKJEU5, 5KJEU8, SKJEU1, SKJEU7, YKJEU1, YKJEU4, YKJEU6, YKJEU, MKMIN6, 17: *W. paramesenteroides* 5KRMA4 (control), 18: *Leuc. citreum* 5KDIA7, 19: *L. saerimneri* GKJEU6.

**Highlights**

- MALDI-TOF MS with 16S rRNA gene sequencing revealed the LAB species in *Kunu-zaki*
- The most dominant LAB species were *Lactiplantibacillus plantarum*, *Weissella confusa*, and *Pediococcus pentosaceus*
- Abundant EPS production in MSFH medium occured among some LAB species in *Kunu-zaki*
- *Weissella confusa* YKDIA1 and YKDIA4 yielded 11.93 and 11.70 g/L dextran in MSFH-5% sucrose
- *Weissella confusa* YKDIA1 increased the viscosity and water holding capacity of *Kunu-zaki*