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# Isolation and identification of probiotic *Lactobacillus* species from traditional drink kunun-zaki fortified with paddy rice and sweet potatoes

Victor-Aduloju A. T.<sup>1\*</sup>, Anyamene C.<sup>2</sup>, Ogbu K. N.<sup>3</sup> and Ishiwu C. N.<sup>1</sup>

<sup>1</sup>Department of Food Science and Technology, Faculty of Agriculture, Nnamdi Azikiwe University Awka, Anambra State, Nigeria.

<sup>2</sup>Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University Awka, Anambra State, Nigeria.

<sup>3</sup>Department of Agricultural and Bioresources Engineering, Nnamdi Azikiwe University Awka, Anambra State, Nigeria.

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The present study was conducted to determine the probiotic properties of the lactic acid bacteria (LAB) isolated from Kunun-zaki processed with different thickeners. Kunun-zaki was processed using 50:50 and 80:20 millet and sorghum. A total of seven isolates that is, *Lactobacillus (Lb.) lactis*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lb. acidophilus*, *Lactobacillus fermentum*, *Lactobacillus delbrueckii* and *Lactobacillus plantarum* were isolated. The isolated LAB showed good growth in the presence of 1% NaCl but none at 10% NaCl except *L. plantarum*. The susceptibility of isolated *Lactobacillus* toward selected pathogenic organisms was determined using standard agar well diffusion method. *Lb. plantarum* was predominant and showed the most significant antimicrobial inhibition against tested pathogenic strains (*Pseudomonas aeruginosa*, *Staphylococcus haemolyticus* and *Klebsiella pneumoniae*) tested followed by *Lb. casei*. The results of this study showed that LAB species isolated from Kunun-zaki processed fulfill the most common criteria of probiotic bacteria. Therefore, numerous probiotic products can be developed from Kunun-zaki.

**Key words:** Probiotics, kunun-zaki, *Lactobacillus* spp., antimicrobial inhibition.

## INTRODUCTION

*Lactobacillus* spp. represents a highly diverse group of Gram-positive micro-aerophilic bacteria that microscopically appear as long to short rods (MacFaddin, 2000). Species within this genus are generally catalase-negative, either homo or heterofermentative with regard

to hexose metabolism (Hasan and Frank, 2001). Certain species of lactobacilli are important and are gaining increasing attention in food fermentation industry because of their biotechnologically interesting properties (Roy et al., 2000). Based on their generally regarded as

\*Corresponding author. E-mail: yemtop2013@gmail.com.

safe (GRAS) status, lactobacilli have been extensively studied for their molecular biology in order to improve their specific beneficial characteristics (Daly and Davis, 1998). The largest group of probiotic bacteria in the intestine is lactic acid bacteria (LAB) (Diplock et al., 2001).

Probiotics are live microorganisms that are similar to beneficial microorganisms found in the human gut and have emerged as a major balancing factor influencing gastrointestinal physiology and function. In the food industry, LAB is widely used as starter cultures and has been recognized to be part of human microbiota (Holzapfel and Wood, 1995). The criteria for the *in-vitro* selection of lactobacilli to be used as health-promoting probiotic ingredients in food and pharmaceutical preparations include antibiotic tolerance as well as the production of lactic acid that inhibits the growth of other microorganisms which allow them to be established in the intestinal tract (Oskar et al., 2004).

LAB is very significant to human health due to the production of some antimicrobial substances and ability to inhibit pathogenic bacteria (Flórez et al., 2005). Furthermore, the bacteria are also used as a starter culture in the production of various foods. Certain *Lactobacillus* strains are considered important owing to their role in various foods and feed fermentations, production of many important metabolites and owing to their role in the prevention of food spoilage. Furthermore, they play a role in combating intoxication and infection by acting as antagonists against other pathogens through the production of antimicrobials and bacteriocin (Hirano et al., 2003; Oyetayo, 2004).

Kunun-zaki is one of the most highly consumed cereal-based non-alcoholic, non-carbonated beverages in Nigeria (Ayo et al., 2004). It is a fermented non-alcoholic cereal beverage whose popularity is due to its characteristic sweet-sour taste typical of LAB fermented foods of African origin (Efiuvwevwere and Akoma, 1995). Cereals used for Kunun-zaki include: sorghum (*Sorghum bicolor*), millet (*Pennisetum typhoideum*), maize (*Zea mays*), rice (*Oryza sativa*), acha (*Digitalis exilis*) or wheat (*Triticuma estivum*) and other cereals could be used in non-composite proportions (Ayo-Omogie and Okorie, 2016).

Kunun-zaki is normally flavoured with a combination of spices which includes ginger (*Zingiber officinale*), cloves (*Eugenia aromatica*), black pepper (*Pipper guinese*), cinnamon (*Xylopia acthiopica*) and together with saccharifying agents such as paste of sweet potato tubers, malted rice, malted sorghum, crude extract from dried *Cadaba farinose* stems are also added (Adebayo et al., 2010).

Research has been done on the production of Kunun-zaki and many strains of probiotic bacteria have been isolated from different sources. However, not much studies has been carried out on isolation of probiotic *Lactobacillus* strains from Kunun-zaki processed with

sweet potatoes and paddy rice thickener, so there is little or no information on it. The aim of this study was to isolate and identify probiotic *Lactobacillus* species from traditional drink Kunun-zaki fortified with different thickeners.

## MATERIALS AND METHODS

### Collection of raw materials for Kunun-zaki production

Millet (*Pennisetum glaucum*), sorghum (*Sorghum bicolor*), ginger (*Zingiber officinale*), red pepper (*Capsicum annum*), clove (*Eugina caryophyllata*) and paddy rice (*Oryza sativa*) were all purchased from Eke Awka market, Awka South Local Government, Anambra State, Nigeria. These grains were sorted, cleaned and stored in plastic containers at ambient temperature before being used.

### Collection of pathogenic microorganisms

Three pathogens (*Staphylococcus haemolyticus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) were collected from Department of Microbiology, Chwukwuemeka Odumegwu Ojukwu Teaching Hospital, Awka, Anambra State, Nigeria. The isolates were confirmed by morphological and biochemical tests according to Cooper and Lawrence (1996). The isolates were sub-cultured on nutrient agar plates and incubated for 24 h at 35-37°C. The colonies were picked and stored on slants at 4° C until when needed.

### Preparation of sweet potatoes

Sweet potatoes (*Ipomea batatas*) was purchased from Government House Market Awka, Nigeria. The potatoes were washed, sliced into 2 cm and sundried for 5 days then ground into powder.

### Preparation of ground malted rice paste

The 250 g of paddy rice to be used was washed with tap water and soaked in 500 ml of tap water (1:2 w/v) for 12 h and then drained. The drained grains were couched by covering them with a moist cloth for 4 to 5 days at ambient temperature (30°C) to germinate and then dried in the sun for 3 days. The dried malted rice was washed and ground into a paste.

### Preparation of Kunun-zaki

Kunun-zaki was prepared from 50:50 and 80:20% millet and sorghum with modification on ingredients according to Adelekan et al. (2013). 500 g of cereal grains were washed with potable water, drained and steeped in 1000 ml of tap water (1:2 w/v) in a bucket for 8 h after which the grain was washed and mixed with 60 g of spices (ginger 40 g, clove 10 g, red pepper 10 g), these were washed and then ground to paste (Figure 1). The slurry was divided into two unequal portions (1:3 w/w). The larger portion was gelatinized by the addition of boiling water (1:1v/v) in a plastic container and stirred vigorously (2 to 3 min) following which it was cooled to about 50°C. The slurry was allowed to sediment and ferment for 12 h. The fermented samples were sieved using a clean muslin cloth (mesh 350 µm). The supernatant liquid was decanted and the filtrate (Kunun-zaki) was packed in the sterile container for subsequent analysis (Figure 1). The paddy rice and sweet potatoes based Kunun-zaki were produced differently by applying the aforementioned procedures. Kunun-zaki produced without any

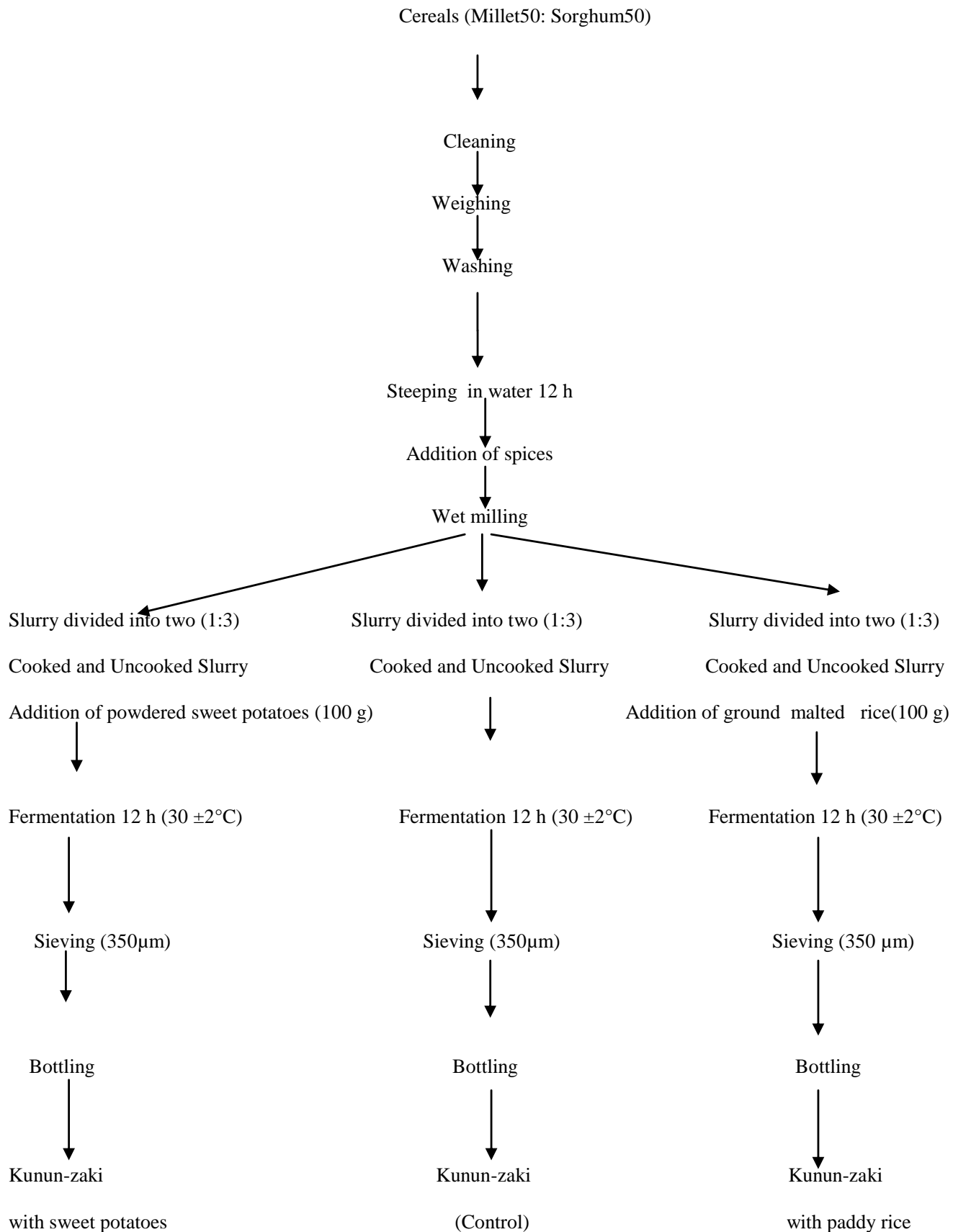


Figure 1. Flow chart for the production of different Kunun-zaki samples.

thickener served as control. Figure 1 showed the preparation of Kunun-zaki.

### Isolation of lactic acid bacteria

The bacteria *Lactobacillus* spp. were isolated from Kunun-zaki samples by using modified MRS broth and MRS agar (Tharmaraj and Shah, 2003). Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of *Lactobacillus* spp. (Hartemink et al., 1997). The pH of the media was adjusted to 6.5. All media and glassware's were autoclaved for 15 min at 121°C before use. 1 ml of each sample was separately suspended in 100 ml of MRS broth of pH 6.5 and homogenized. Five-fold dilutions were made from each homogenized sample, and all dilutions were incubated for 24 h at 37°C under an anaerobic condition in the presence of 5 % CO<sub>2</sub>. A loopful of each culture was streaked on to the MRS agar plate and plates were incubated under anaerobic condition at 37°C for 24 h. The single colony of *Lactobacillus* was isolated by observing their colony morphology and some biochemical tests (Gram staining, catalase and motility test), and the culture were maintained in MRS broth at pH 5.5.

### Identification

The isolated bacteria were identified as *Lactobacillus* species by observing their morphological characteristics: Gram staining, motility test, catalase test, indole, oxidase and milk coagulation activities and 1-10% NaCl tolerance test (Issazadeh et al., 2013).

### Sugar fermentation test

MRS broth at pH 6.5 was put into a screw-capped test tube and phenol red (0.01 g per L) was added into the tube as pH indicator (Devriese et al., 1993). After autoclaving, 1 ml of different sterilized sugar solutions (10%) was inoculated into the different tube. Then, 200 µl of an overnight bacterial culture was inoculated into the broth medium and incubated anaerobically at 37°C for 24 h. As a pH indicator, phenol red was included in the medium; acid production changed the medium from its original color to yellow (Manero and Blanch, 1999). After adding the proper amount of broth, Durham tubes were inserted into each culture tube in order to observe gas production (Herrero et al., 1996).

### Screening of isolated *Lactobacillus* species for probiotic properties

#### Tolerance of isolated LAB to acidic pH

The tolerance of the probiotic bacteria to acidic pH was tested *in vitro* as described by Pelinescu et al. (2009). 1 ml of each LAB culture at  $1 \times 10^8$  CFU/ml was inoculated into sterile MRS broth and incubated anaerobically at 37°C overnight, then sub-cultured into fresh MRS broth tubes of pH 2 to 4 (broth was adjusted by a pH meter using HCl and NaOH) and incubated anaerobically at 37°C for 24 h. After incubation, 1 ml inoculums from each tube was inoculated into MRS agar medium using pour plate technique and incubated anaerobically at 37°C for 48 h. The growth (indicated by presence or absence of growth) of the LAB on MRS agar was used to designate isolates as pH tolerant.

#### Sensitivity to temperature

The selected LAB cultures were inoculated into 10 ml sterile MRS

broth and incubated anaerobically at varying temperatures (from 15 to 45°C) for 48 to 72 h. Thereafter, 1 ml inoculums were transferred to MRS agar plates by pour plate method and incubated at 37°C for 48 h. The growth of LAB on MRS agar plates was used to designate isolates as temperature tolerant (Tambekar and Bhutada, 2010).

#### NaCl tolerance

Tested LAB cultures were inoculated into 10 ml sterile MRS broth with NaCl concentration between 1 to 10% and incubated at 37°C for 48 h. Growth was monitored by visual inspection of the test tubes and NaCl tolerance was evaluated after 1 ml was plated using sterile MRS agar, allowed to set and incubated at 37°C for a period of 48 h (Tambekar and Bhutada, 2010). Positive control experiments were made of tubes containing LAB cultures without additional NaCl, while negative control experiments were tubes with added NaCl but without LAB cultures.

#### Assay for antimicrobial activity

Antimicrobial activities of probiotics were determined by the agar well diffusion method as described by Tajehmiri et al. (2014). A 0.2 ml of a 24 h broth culture was aseptically introduced into the sterile petri dishes. The sterilized medium at 45 to 50°C was poured into petri dishes. The agar depth was 4 mm. A 26 ml medium was used for the plate with 90 mm diameter. Wells were made on the agar plates using a sterile cork borer of 5 mm diameter. A 100 µl of the supernatants of isolated probiotics were placed into each well. A negative control was 100 µl of the broth without organisms. The culture plate was incubated at 37°C for 48 h, and the resulting zones of inhibition were measured using a ruler calibrated in millimeter. Each experiment was replicated three times and the results were expressed as average values. Isolates which gave an inhibition zone bigger than 10 mm was determined to have antimicrobial activity.

#### Statistical analysis

Data were subjected to analysis of variance (ANOVA) using Minitab 16.0 statistical software (Minitab Inc., State College, PA, USA), and they were characterized descriptively as means  $\pm$  standard deviation. Statistically, significant means were separated using Duncan Multiple Range test (DMRT). The significance level adopted was  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

### Isolation and identification

Seven *Lactobacillus* bacterial isolates were obtained from five samples of Kunun-zaki. The organisms include *Lactobacillus lactis*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus delbrueckii* and *Lactobacillus plantarum*. Microscopically, the isolates were: Gram positive, rod-shaped, oxidase negative, catalase negative, non-motile, indole- negative, starch hydrolysis negative and absence of endospores. The carbohydrates fermentation patterns of the isolates and their characteristics are listed in Table 1. This is in agreement with the study of Adelekan et al. (2013) and Okoronkwo

**Table 1.** Physiological and biochemical properties of *Lactobacillus* species isolated from Kunun-zaki.

Variable	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7
Gram reaction	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-
pH3	+	+	+	-	+	+	+
pH5	+	+	+	+	+	+	+
15°C	+	+	-	+	+	-	+
45°C	+	+	+	+	+	+	+
CO <sub>2</sub> from glucose	+	-	-	-	+	-	+
Production of acid	-	-	-	-	+	-	+
Glucose	+	+	+	+	+	+	+
Lactose	+	+	-	+	+	+	+
Sucrose	+	-	+	+	+	-	+
Mannitol	+	+	-	-	+	-	+
Sorbitol	-	+	-	-	+	-	+
Gala	+	+	-	+	+	-	+
Arabinose	+	+	+	+	+	-	+
Probable identity	<i>L. lactis</i>	<i>L. casei</i>	<i>L. brevis</i>	<i>L. acidophilus</i>	<i>L. fermentum</i>	<i>L. delbrueckii</i>	<i>L. plantarum</i>

+ = Positive reaction; - = Negative reaction.

**Table 2.** LAB isolated from Kunun-zaki processed with natural thickeners.

Isolate	Source of isolation	Significant LAB isolated
1	Millet- Sorghum at 50:50%	<i>Lactobacillus lactis</i>
2	Millet- Sorghum+ Paddy Rice at 50:50%	<i>Lactobacillus casei</i>
3	Millet- Sorghum + Sweet Potatoes at 50:50%	<i>Lactobacillus brevis</i>
4	Millet- Sorghum + Sweet Potatoes at 50:50%	<i>Lactobacillus acidophilus</i>
5	Millet and Sorghum at 80:20%	<i>Lactobacillus fermentum</i>
6	Millet and Sorghum at 80:20%	<i>Lactobacillus delbrueckii</i>
7	Millet- Sorghum + Paddy Rice at 80:20%	<i>Lactobacillus plantarum</i>

(2014). The identified LAB are organisms which are naturally present in the human gut also they are the organisms that cause fermentation in Kunun-zaki (Oluwajoba et al., 2013). Table 2 showed that *L. fermentum* and *L. delbrueckii* were isolated from Kunun-zaki made from millet and sorghum at 80:20%. The colonies of *Lactobacillus* isolates appeared rough, dull white, 0.1 to 0.5 mm in diameter, and demonstrated medium to short rods.

### Sugar fermentation

Isolate 7 tentatively identified as *Lactobacillus plantarum* fermented all the sugars used. *L. lactis*, *L. brevis*, *L. acidophilus* and *L. delbrueckii* did not produce gas from glucose and other sugars, and also showed variation in sugar fermentation patterns. Sorbitol was only

fermented by three isolates.

### Sensitivity to temperature and pH

The growth of the *Lactobacillus* species as presented in Table 1 showed that all the isolates were able to grow at high temperature but *L. brevis* and *L. delbrueckii* showed no growth at 15°C. According to Ibourahema et al. (2008), the bacterial capability to grow at high temperature is a good characteristic as it could be interpreted as indicating an increased rate of growth and lactic acid population. LAB are acidophilic which means they are tolerant to low pH (Klaenhammer and Kullen, 1999). However, this needs to be differentiated from a condition of high concentration of free acids because the free acids may cause growth inhibitors (Mohd and Tan, 2007).

**Table 3.** Sodium chloride tolerance of isolated *Lactobacillus* spp..

LAB	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
A	++	+	+	+	+	+	+	+	+	+
B	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus lactis</i>	++	+	+	+	+	+	+	+	+	-
<i>Lactobacillus casei</i>	++	+	+	+	+	+	+	+	+	-
<i>Lactobacillus brevis</i>	++	+	+	+	+	+	+	+	+	-
<i>Lactobacillus acidophilus</i>	++	+	+	+	+	+	+	+	+	-
<i>Lactobacillus fermentum</i>	++	+	+	+	+	+	+	+	+	-
<i>Lactobacillus delbrueckii</i>	++	+	+	+	+	+	+	+	+	-
<i>Lactobacillus plantarum</i>	++	+	+	+	+	+	+	+	+	+

++ = Good growth; + = visible growth; - = no growth. A=Positive control (Tubes with lactic acid bacteria cultures without NaCl); B=Negative control (Tubes with NaCl but without cultures); LAB= Lactic acid bacteria.

**Table 4.** Inhibition zones (mm) induced by lactic acid bacteria against the pathogenic bacteria.

LAB isolates	Inhibition zone (mm)		
	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus haemolyticus</i>	<i>Klebsiella pneumoniae</i>
<i>Lactobacillus lactis</i>	8.43±0.05 <sup>f</sup>	7.57±0.02 <sup>g</sup>	6.82±0.03 <sup>g</sup>
<i>Lactobacillus casei</i>	13.57±0.02 <sup>b</sup>	14.03±0.03 <sup>b</sup>	11.83±0.02 <sup>b</sup>
<i>Lactobacillus brevis</i>	8.69±0.04 <sup>e</sup>	8.46±0.06 <sup>e</sup>	8.80±0.04 <sup>c</sup>
<i>Lactobacillus acidophilus</i>	9.71±0.55 <sup>c</sup>	9.42±0.06 <sup>c</sup>	8.46±0.02 <sup>d</sup>
<i>Lactobacillus fermentum</i>	9.46±0.04 <sup>d</sup>	8.14 ±0.01 <sup>f</sup>	7.25±0.04 <sup>e</sup>
<i>Lactobacillus delbrueckii</i>	5.15±0.02	9.23±0.01 <sup>d</sup>	7.04±0.06 <sup>f</sup>
<i>Lactobacillus plantarum</i>	13.86±0.03 <sup>a</sup>	14.25±0.03 <sup>a</sup>	13.19±0.03 <sup>a</sup>

Each data is the mean of triplicate determination with standard error.; Different superscripts within the same column with each test are significantly different (P≤5).

### Tolerance to NaCl

In this study, all the isolated probiotic candidates were able to grow at 1 to 9% NaCl, whereas fairly grow at 7% but completely failed at 8 to 10% NaCl (Table 3). The tolerance of all the isolates to high NaCl concentration (4 to 9%) further indicated their potential to survive the harsh conditions and bile salt of the intestine (Cullimore, 2000; Topisirovic et al., 2006).

### Antimicrobial activity of lactic acid bacteria

The results of probiotics antimicrobial effect on selected pathogenic organisms are shown in Table 4. The comparison of the inhibitory zones caused by the seven strains of probiotics (*L. lactis*, *L. casei*, *L. brevis*, *L. acidophilus*, *L. fermentum*, *L. delbrueckii* and *L. plantarum*) showed that the antimicrobial effect of *L. plantarum* and *L. casei* was significantly different from other strains. *L. plantarum* had the highest zone of inhibition on all the pathogenic organisms (Table 4). *L. delbrueckii* exhibited lowest zone of inhibition on *Pseudomonas aeruginosa* (5.15±0.02 mm). Antibacterial activity of *L. lactis* against *Staphylococcus haemolyticus*

and *Klebsiella pneumoniae* showed lowest zones of inhibition (Table 4). The observed variation in the inhibition of the test pathogens by the LAB is an indication that the organisms possess varying abilities to exert antimicrobial effects on pathogens and this corroborates the study of Azcarate-Peril et al. (2004) that antimicrobial effect exerted by lactic acid bacteria are strain specific. In general, tolerance to sodium chloride salts has been considered a condition for colonization and metabolic activity of bacteria in the host intestine (Anadon et al., 2006). The low resistance of *Lactobacillus delbrueckii* toward *Pseudomonas aeruginosa* may be an indication of their potential to survive the temperature of the human gut since temperature is an important requirement for bacterial growth, and the selected temperature range was chosen to simulate the normal human body temperature. This factor is very important in determining the effectiveness of probiotics since growth and viability during storage and use is one of the important determining factors for the functionality of probiotics (Tambekar and Bhutada, 2010) (Tables 1 to 4).

### Conclusion

*L. plantarum* has been shown to be the most effective

probiotic which exerted the highest antimicrobial effect against the test pathogens. Lactic acid bacteria from Kunun-zaki may act as a reservoir of antimicrobial-resistance genes. These bacteria could act as bio-therapeutic microorganisms and might be good probiotic drink.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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