British Microbiology Research Journal
4(12): 1311-1324, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Antibiotic Susceptibility and Intestinal Epithelial Cell Adhesion Pattern of Homo Fermenting Lactic Acid Bacteria (LAB) Isolated from Kunu-Zaki, a Spontaneously Fermenting Nigerian Cereal Beverage

S. O. Oluwajoba[1,2*], F. A. Akinyosoye[1] and V. O. Oyetayo[1]

[1]Department of Microbiology, Federal University of Technology, Akure, Nigeria.
[2]Department of Biological Science, Yaba College of Technology, Yaba, Lagos, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author SOO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors FAA and VOO managed the analyses of the study. Author SOO managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

Received 8th April 2014
Accepted 28th June 2014
Published 20th July 2014

ABSTRACT

Aim: To determine the antibiotic reaction and adhesion pattern of antimicrobial homo-fermenting LAB strains in the fermenting slurries of kunu zaki.
Study Design: ANOVA. Inhibition of indicator lawn used ≥10mm inhibition as antibiotic susceptible. Adhesion was measured by staining and quantifying recorded as percentage and index values.
Place and Duration of Study: Department of Microbiology, Federal University of Technology Akure and Biotechnology Unit, Federal Institute of Industrial Research, Oshodi, Nigeria between June, 2012 and December, 2012.
Methodology: Kunu-zaki drinks were produced using spontaneously fermenting cereal

**Corresponding author: Email: solakunmi@yahoo.com;*

grains of Digitaria exilis (acha), Sorghum bicolour (sorghum) and Pennisetum americanum (millet) in composite and non-composite proportions. LAB isolates were obtained on MRS agar. Homo-fermenting isolates were identified to species level using the API 50 CHL test kit. Antibiotic sensitivity testing on the identified isolates followed the modified standard Kirby-Bauer procedure on MRS agar (pH 7.4) using the disc diffusion technique with selected antibiotics. For quality control of the antibiotics, sensitive reference strains *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 obtained from the Nigeria Institute of Medical Research were used. Adhesion and antimicrobial properties were determined using standard method.

Results: Antimicrobial substances produced by the eight LAB isolates inhibited the growth of four selected human pathogens in vitro. All eight LAB isolates were resistant to amoxicillin, gentamycin and ciprofloxacin. *L. plantarum*1[26], *L. paracasei* subsp *paracasei*3[39] and *Pediococcus damnosus*[32] were resistant to erythromycin whilst all others were susceptible. *L. plantarum*1[26] and *L. paracasei* subsp *paracasei*3[39] were resistant to all antibiotics tested. All LAB isolates demonstrated high in-vitro intestinal epithelial cell adhesion potential.

Conclusion: LAB antimicrobial activity may not be affected if kunu zaki is consumed simultaneously with these antibiotic therapies. However, if these LAB strains are intended for use as kunu-zaki starter cultures, it is important that they should be further carefully examined for inability to transfer antibiotic resistance genes to food pathogens.

Keywords: lactic acid bacteria; antimicrobial; antibiotics; susceptibility; resistance.

1. INTRODUCTION

The lactic acid bacteria (LAB) are easily identified as Gram positive bacteria, non-respiring, non-spore forming, cocci or rods [1]. Lactic acid is the major end product of the fermentation of carbohydrates, though some fermentation produces other substances in addition to lactic acid. Fermentation of sour dough bread, sorghum beer, milks, cassava (to produce *gari* and *fufu*), *ogi*, *kenkey, injera, obiolor* are all examples of African fermented foods involving lactic acid bacteria [1]. The major genera of lactic acid bacteria include the *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* species and even though a few more have been identified, they play a minor role in lactic fermentations [1]. Lactic acid bacteria are usually found in decomposing plants and lactic products. Lactic acid is the major metabolic end product of the carbohydrate fermentation. LAB are a large group of fermentative, anaerobe aero-tolerant microorganisms which are usually present in the gut of humans and other animals, raw vegetables, meat and meat products, and cereal [1]. In animals, their numbers may vary with the species, the age of the host, or the location within the gut [2]. In the food industry, lactic acid bacterial strains are widely employed either as starter cultures or as non-starter lactic acid bacteria. Furthermore, owing to their probiotic properties, several LAB strains have found use as adjunctive cultures in foods and feed [3]. Increasing commercial interest for functional food containing probiotics, has increased scientific interests in these products. Lately, the researches in the food biotechnologies have centered on careful isolation and selection of strains of *Lactobacillus* having antimicrobial properties that might both ensure the microbiological safety of the food as well as bring benefits to the consumer`s health [4]. One of the anxieties which have evolved over the use of LAB as starter cultures, however, is resistance to antibiotics. Antibiotics are one of the great discoveries against bacterial infections. Unfortunately bacteria can fight back by being resistant towards antibiotics. From a human perspective, resistance to antibiotics is an un-desirable ability of microorganism. Antibiotic resistance is the ability of a microorganism to

remain unaffected by an antibiotic. When a microorganism becomes antibiotic resistant, it is difficult to eliminate the infection caused by these microorganisms if and when it happens. Microorganisms can become resistant to antibiotics either by inactivating the drug, by altering the target site, by altering the metabolic pathway, or by reducing drug accumulation [5]. In recent times, antibiotic resistance in bacteria has become a public concern issue. This is because a patient could develop antibiotic resistance by contacting a resistant microorganism or the emergence of a microorganism in the patient's body when treatment with antibiotic begins [6]. Even though a health beneficial microorganism such as LAB with antibiotic resistance may have the advantage of not being affected by the antibiotic when a consumer takes it together with the antibiotic, however, such an organism with antibiotic resistance could eventually become an opportunistic pathogen. This could be highly detrimental [7], to the infected host and the transfer of antibiotic resistant genes from the health beneficial food LAB to food pathogens may occur. Some species of LAB commonly used in the food industry or naturally occurring in raw food materials have been found to be resistant to glycopeptides antibiotics [8]. Genes conferring resistance to several antimicrobials (including chloramphenicol, erythromycin, streptomycin, tetracycline, and vancomycin) located on transferable genetic elements (plasmids and transposons) have already been characterized in lactococci [7], and lactobacilli [9], from foods. Frequency of spontaneous mutation to kanamycin and streptomycin was reported to be very high for an appreciable number of lactobacilli [5]. Currently there are lots of data on the prevalence of antibiotic resistance and the mechanisms implicated in clinical bacteria [9]. It is therefore important that strains intended for use in the food systems should also be carefully examined for antimicrobial susceptibility/resistance. Kunu-zaki is a traditional beverage made from spontaneously fermenting cereals. The beverage originated from Northern Nigeria but has found wide acceptability in all parts of the country. Cereals used for kunu-zaki include sorghum, millet or maize in non-composite proportions. Regardless of the cereal used, kunu-zaki wild fermentation is largely dominated by Lactic acid [10,11]. The spontaneous fermentation production process however usually results in kunu zaki of inconsistent quality from batch to batch of each production. The use of starter cultures, which would produce a uniform fermented kunu-zaki product quality remains yet unexplored. Yet this drink has become very popular in a country referred to as the most populous African nation. The hypothesis behind this present work therefore is that it would be possible to select strains with antimicrobial properties which can be used as starter cultures for kunu-zaki. The aim of this current study was to evaluate the antibiotic reaction pattern and the adhesion ability of antimicrobial homo-fermenting LAB strains present in fermenting slurries of kunu zaki. This would be helpful in determining if the LAB would be affected if a kunu-zaki drinker is on an antibiotic therapy. The information obtained from this study could contribute to the potential use of these LAB isolates in the food and pharmaceutical industries.

2. MATERIALS AND METHODS

2.1 Laboratory Production of Kunu-Zaki

Sorghum (*Sorghum bicolour)*, millet, (*Pennisetum americanum)* and hungry rice (locally known as Fonio or Acha) *Digitaria exilis* grains were obtained from the Nigeria Cereal Research Institute in Ibadan, Oyo State, Nigeria. The grains were cleaned, weighed and washed before steeping in distilled water.200 grams of cereal grains were used for the kunu-zaki production. A control experiment was set up with distilled water without the grains. For the kunu-zaki made from composite grains, an equal weight of grains was used for each

part. The laboratory production method was done according to the traditional process of kunu-zaki fermentation reported by Adeyemi and Umar [12] with slight modifications.

2.2 Isolation of the Lactic Acid Bacteria

The isolation of the lactic acid bacteria was done following the pour plate technique.

Samples were obtained directly from the 72 hours fermenting mash of the kunu-zaki made from each cereal type. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory. Once delivered to the laboratory, they were kept under refrigeration temperature until ready for analysis.

The Pour plate technique was used to isolate the organisms. The kunu-zaki samples were used directly, diluted to 10^{-1}, 10^{-2} and 10^{-3} using sterile peptone water. 1 ml aliquot of the samples and dilutions were plated into MRS agar (pH 6.2 and pH5.5). The plates were incubated at 37ºC for 3 days under anaerobic conditions (in an anaerobic jar using Oxoid anaerogen compact). The use of the medium was to isolate and enumerate the lactobacilli present in the sample. After incubation, individual colonies were selected and transferred into sterile broth mediums. The streak plate technique was used to obtain pure colonies.

2.3 Morphological, Physiological and Biochemical Examination of LAB

The pure colonies isolates were examined according to their colony morphology, catalase reaction and gram reaction. The Gram positive and catalase negative cocci and bacilli colonies were stored in glycerol as lactic acid bacteria. Morphological, physiological and biochemical examination of the isolates were determined by the standard procedure of gram staining, catalase test and test for CO_2 gas production according to methods offered by Bulut [13].

2.4 Catalase Test

Lactic acid bacteria do not contain the catalase enzyme and are designated catalase negative. The catalase test was performed on the pure culture isolates in order to confirm their catalase reaction.

Overnight cultures of the pure culture isolates were grown in MRS broth at 37ºC under anaerobic conditions. After 24 hours, 3% hydrogen peroxide solution was dropped into 1 ml of the overnight culture. The isolates, which did not give gas bubbles, were chosen as catalase negative [13].

2.5 Gram Staining

The gram reaction of the isolates was determined by light microscopy after gram staining. LABS are gram positive and appear purple-blue color under the microscope after gram staining.

2.6 Homo/Hetero-Fermentative Property

The catalase negative, gram positive isolates were tested for CO_2 production from glucose to determine which of the identified isolates were either homo fermentative or hetero

fermentative. Citrate lacking MRS broths with inserted inverted Durham tubes were prepared and inoculated with 1% overnight fresh cultures. Then the test tubes were incubated at 37ºC for 5 days. Gas production in the Durham tubes evidenced by displacement of the broth was observed during the 5 day incubation period. Evidence of gas production was recorded as positive indicating hetero-fermentation [13].

2.7 Temperature and NaCl Tolerance Test

To determine tolerance to different temperatures, MRS containing bromo-cresol purple indicator was prepared and 5mls transferred into tubes. Fifty µl of overnight cultures was inoculated into the tubes and incubated for 7 days at temperatures 10ºC, 15ºC, 45ºC. For each incubation temperature, the cell growth was observed by the color change of the indicator purple to yellow and recorded as positive (+). For the NaCl tolerance test, two different NaCl concentrations (4.5% and 6.0%) were added to MRS broth containing the indicator. The tubes were inoculated with 1% overnight cultures and incubated at 37ºC for 7 days. Cell growth was indicated by color change and recorded as positive (+) [13].

2.8 Identification with API 50CHL

The means of identification used was the API 50 CHL medium intended for the identification of the genus Lactobacillus and related genera. A suspension was made in the medium with the microorganism to be identified and each tube of the strip was inoculated with the suspension. During incubation, the carbohydrates fermented to acids which produced a decrease in the pH detected by the change in color of the indicator. The results made up the biochemical profile which was used by the apiweb^{TM} identification software BioMériux (Ref.40011) to identify the strain. Strains were identified to species level with specified accuracy percentages.

2.9 Determination of Antibiotic Susceptibility

Standardised culture suspensions (0.5 McFarland standard, equivalent to cell density of 10^8 cfu/ml) was spread evenly on the surface of the MRS agar plate (Oxoid) using a sterile cotton swab. The inoculated plate was allowed to dry before placing the diffusion discs containing antibiotics onto the surface of the inoculated agar plates. Susceptibility of the eight isolates to 5 types of antibiotics was performed by the Kirby Baeur disc diffusion method as used by Rojo-Bezares et al. [14]. Commercially available antibiotics discs (Oxoid) containing amoxicillin (25µg), erythromycin (10µg), ciprofloxacin (10µg), cotrimoxazole (25µg) and gentamycin (10µg) were used. Precaution was taken to ensure uniform contact between the antibiotic disc and agar plate. After 36 hours incubation at 30-32ºC under anaerobic conditions, inhibition zone diameters were measured. Inhibition zone diameters were measured inclusive of the diameter of the discs. Results were expressed as sensitive S, intermediate, I, and resistant, R, respectively according to the cut-off points given by the manufacturer. For quality control of the antibiotics used during the study, sensitive reference strains *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 obtained from the Nigerian Institute of Medical Research (NIMER), Lagos Nigeria were used.

2.10 Adhesion Assay

This test was done according to the method of Mardh and Westron [15] with slight modifications as reported by Otero and Nader-Macias [16]. Apparently healthy guinea pigs

were obtained from the National Food and Drug Administration and Control (NAFDAC), Lagos, Nigeria. Intestinal epithelial cells were collected by scraping the intestinal wall of the guinea pigs with a brush and suspending at pH 7.0 in MEM (Eagles Minimal Essential Media). The suspension was kept under refrigeration until the adhesion assay. For the adhesion assay, bacterial suspension was standardised by harvesting the LAB isolate from a 12 hour pure culture and washing twice with saline (0.8% NaCl) solution, once with MEM (pH 7.0) and re-suspension in MEM to obtain a final concentration of 10^7 cfu/ml (OD_{540}=0.15). The bacterial suspension (500µl) was then added to 500µl of guinea pig intestinal cell suspension and incubated at 37^0C for 1 hour under low agitated conditions (35 rpm). The control was prepared by substituting the bacterial suspension with only MEM. In order to remove non-adherent bacteria, tubes were centrifuged for 10 minutes at 800 rpm and the supernatant discarded. The pellets were re-suspended in 1ml MEM and washed three times under the same conditions. Bacterial binding to the epithelial cells was examined by light and phase contrast microscopy using differential staining [16].

The percentage adhesion was expressed as:-

$$\%\ adhesion = \frac{No\ of\ intestinal\ epithelial\ cells\ with\ bacteria\ adhered}{Total\ No\ of\ intestinal\ epithelial\ cells}\ x\ 100$$

The adhesion index was expressed as:-

$$Adhesion\ index = \frac{Total\ No\ of\ bacteria\ attached\ to\ epithelial\ cells}{No\ of\ cells\ with\ adhered\ bcteria}$$

2.11 Antimicrobial Activity

Antimicrobial activity was assessed using the method of Rojo-Bezares et al. [14]. After 18 hours' incubation; active cultures were spotted on the surface of MRS agar plates. The plates were incubated to grow the cultures for 24 hours at 37ºC under anaerobic conditions. Overnight indicator reference pathogen inoculated to soft agar containing 0.7% agar was overlaid on the MRS plates. Indicator organisms used were *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *P. aeuruginosa* ATCC27853, *and E. coli* ATCC 25922. Indicator organisms were obtained from the Nigeria Institute of Medical Research (NIMER), Lagos, Nigeria. The plates were incubated according to the appropriate condition for the indicator organism. At the end of incubation, the inhibition zone around the spotted isolates was measured. Isolates which gave an inhibition zone bigger than 1mm were determined to have antimicrobial activity. Data on the zone of inhibition obtained against the different bacterial pathogens was statistically analysed and significance evaluated at 5% level.

3. RESULTS

3.1 Production of Kunu-Zaki Drinks

The appearance of kunu-zaki, along with its acid taste is one of the reasons why the drink has remained attractive to consumers (Table 1). The low pH in fermented foods is as a result of the production of lactic acid, the end product of the metabolism of the principal fermenting organisms. Because of their low pH, fermented foods are judged to be safe. The pH of the kunu-zaki types produced in this present study ranged from 4.04-5.41. This concurs with values that have been obtained [17].

Table 1. Laboratory production of kunu-zaki

Kunu-zaki type	Appearance of beverage	Final pH
Sorghum-Millet	Light chocolate brown	5.12
Acha-Sorghum	Milky brown	4.04
Sorghum	Deep brown	5.08
Millet	Milky white	5.41

3.2 Cultural Characteristics of Lactic Acid Bacteria

Cultural characteristics typical of lactic acid bacteria (Table 2) and subsequent identification of the LAB isolated from the different kunu zaki types gave their identification accuracy ranging between 99.3-99.9percent. The fermentation strips were read with the API web^{TM} BioMerieux (France) software which gave confidence as the percentage accuracy indicated a 'very good" identification to "excellent" identification.

3.3 Physicochemical Properties of LAB Isolates

Physicochemical properties of the lactic acid bacteria identified indicated that *Pediococcus pentosaceus* 2^{27} was able to grow at all conditions of temperature and NaCl concentrations tested (Table 3). It is important to study the ability of homo-fermenting LAB to grow at different temperatures [18]. From this study, *Pediococcus pentosaceus* 2^{27} and *L. plantarum1*^{26} were both able to grow at 45ºC. This would make both LAB isolates likely candidates as starter cultures for the large scale production of kunu-zaki under controlled fermentation conditions. All LAB isolates with the exception of *Pediococcus damnosus* were able to grow at 4.5% NaCl concentrations. The introduction of NaCl is also sometimes used in African fermentations to halt fermentation processes [18]. In the event of choice of starter cultures for use in controlled fermentations, ability to withstand NaCl concentrations becomes important [3].

3.4 Adhesion Properties of LAB Isolates

The highest adhesion in this study was demonstrated by *L.paracasei subsp paracasei1*^{6} (Table 4). Adhesion to the GI tract has been widely used as a criterion for the selection of health benefitting lactobacilli [19]. Many bacteria use their ability to adhere as a strategy to maintain stability in their numbers. Some studies have shown that the high capability of adhesion of a certain microorganism indicates that nutrients can be competed for with a higher efficiency than non-adherent bacteria and in this way adherent bacteria can out-number the non-adherent ones. The adhesion to epithelial surfaces is a critical step in the colonization of *Lactobacillus* in the intestine and one of the suggested mechanisms by which they could protect the intestines from the colonization of pathogens is by their competition for the receptor sites for adhesion. The entire homo-fermenting LAB investigated in this study gave high adhesion capabilities *in vitro*. This agreed with the work of Otero and Nader-Macias [15].

Table 2. Characteristics of the LAB isolates

Code	Cultural characteristics	Cell morphology	Gram reaction	Catalase reaction	CO_2 production	API web™ software Identification	% ID
32	Pin point, cream white, entire edge, and smooth, raised	Cocci	+	-	-	*Pediococcus damnosus*	99.8
33	Pin point, cream white, entire edge, smooth, raised	Rods	+	-	-	*Lactobacillus plantarum1*	99.9
6	Pin point, cream white, entire edge, smooth, raised	Rods	+	-	-	*Lactobacillus paracasei subsp paracasei1*	99.7
29	Pin point, cream white, entire edge, smooth, raised	Rods	+	-	-	*Lactobacillus paracasei subsp paracasei2*	99.3
26	Pin point, cream white, entire edge, smooth, raised	Rods	+	-	-	*Lactobacillus plantarum1*	99.9
39	Pin point, cream white, entire edge, smooth, raised	Rods	+	-	-	*Lactobacillus paracasei subsp paracasei3*	99.8
27	Pin point, cream white, entire edge, smooth, raised	Cocci	+	-	-	*Pediococcus pentosaceus 2*	99.9

Table 3. Physicochemical properties of identified LAB

Name of LAB isolate	Homo / Hetero fermenter	Growth at 10ºC	Growth at 15ºC	Growth at 45ºC	Growth in 4% NaCl	Growth in 6.5% NaCl
Lactobacillus plantarum1[33]	HM	+	+	--	+	--
Pediococcus pentosaceus2[27]	HM	+	+	+	+	+
Pediococcus damnosus2	HM	+	+	--	--	--
Lactobacillus paracasei subsp para1[6]	HM	+	+	--	+	+
Lactobacillus plantarum1[26]	HM	+	+	+	+	--
Lactobacillus paracasei subsp para2[29]	HM	+	+	--	+	+
Lactobacillus paracasei subsp paracasei3[39]	HM	+	+	--	+	+
Lactobacillus plantarum1[12]	HM	+	+	--	+	--

+ = *Positive***;--=** *Negative**; HM = Homo-fermenter*

Table 4. Adhesion assay of LAB to intestinal epithelial cells of guinea pigs

Name of LAB[CODE]	Adhesion (%)	Adhesion index	Kunu-zaki fermenting cereal
P. damnosus2 [32]	80	15	Sorghum-Millet
L. plantarum1 [33]	65	18.4	Sorghum-millet
L. paracasei subsp paracasei1 [6]	100	22	Sorghum-Millet
L. paracasei subsp paracasei2 [29]	55	14.5	Acha-sorghum
L. plantarum1 [26]	80	15	Sorghum
L. paracasei subsp paracasei3 [39]	75	6.8	Sorghum-Millet
P. pentosaceus2 [27]	60	12	Millet
L. plantarum1 [12]	70	24	Sorghum-Millet

Table 5. Inhibition zones (mm) of homo-fermenting LAB against selected human pathogens

	Zones of inhibition (mm) Reference pathogens			
LAB significant taxa	***E. coli***	***S. aureus***	***P. aeruginosa***	***E. faecalis***
L. plantarum1 [33]	$21.00\pm1.00^{\text{def DEF}}$	$24.17\pm0.76^{\text{gDEFGH}}$	$23.17\pm1.26^{\text{efghiDEFGH}}$	$17.83^{C}\pm0.76^{\text{defg}}$
P. pentosaceus2 [27]	$28.00\pm1.00^{\text{jk GHIJ}}$	$22.17\pm0.289^{\text{f EFG}}$	$22.33\pm1.15^{\text{cdefgCDEFG}}$	$17.83^{E}\pm0.29^{\text{defg}}$
P. damnosus [32]	$19.67\pm1.53^{\text{de BC}}$	$20.67\pm0.58^{\text{de BCDEF}}$	$18.67\pm0.71^{\text{abcAB}}$	$0.00^{A}\pm0.00^{a}$
L. paracasei subsp para2 [29]	$11.00\pm1.00^{\text{b B}}$	$22.50\pm0.5^{\text{f EFGH}}$	$23.83\pm0.76^{\text{cdefEFGH}}$	$12.83^{C}\pm0.29^{b}$
L. plantarum1 [26]	$22.83\pm1.76^{\text{gh DEFGHI}}$	$26.27\pm1.00^{\text{hiGHI}}$	$21.07\pm1.10^{\text{ghiBCDEF}}$	$24.17^{HIJ}\pm0.29^{b}$
L. paracasei subsp para1 [6]	$28.00\pm1.00^{\text{jk IJ}}$	$24.00\pm0.76^{\text{gFGH}}$	$25.00\pm1.32^{\text{ijKGH}}$	$14.17^{C}\pm0.29^{I}$
L. paracasei subsp para3 [39]	$23.33\pm2.8^{\text{hiH}}$	$20.00\pm1.00^{\text{dBCDEF}}$	$29.00\pm.00^{\text{bcdeHI}}$	$14.00^{C}\pm0.50^{\text{bcd}}$
L. plantarum1 [12]	$15.00\pm1.0^{\text{cBC}}$	$20.50\pm0.89^{\text{deBCDEF}}$	$20.17\pm0.28^{\text{bcdefBCD}}$	$18.00^{CD}\pm0.52^{\text{efg}}$

Values are means of duplicate readings.
Values with the same superscriptsacross rows in small letters and down columns in capital letters are not significantly different

Table 6. Antibiotic reaction of viable kunu-zaki homo-fermenting LAB

Name of lab I[CODE]	25µg amoxicillin	10µg erythromycin	10µg ciprofloxacin	25µg cotrimoxazole	10µg gentamycin
L. plantarum1 [33]	R	13±0.7	R	12±1.1	R
L. paracasei subsp para1 [6]	R	15±1.4	R	R	R
L. paracasei subsp para2 [29]	R	7±1.1	R	R	R
L. plantarum1 [26]	R	R	R	R	R
L. paracasei subsp para3 [39]	R	R	R	R	R
P. pentosaceus2 [27]	R	13±1.1	R	9±2	R
L. plantarum1 [12]	R	10±1.1	R	R	R
P. damnosus [32]	R	R	R	14±1	R
S. aureus	R	R	20±2	17±1	R
E. coli	8±1.1	R^{NIT}	22±1	R	12±0.3

Values (mm) represent growth inhibition zones, Values are means of duplicate readings with standard deviations, R= Resistant; NIT= Nitrofurantoin;

3.5 Inhibition of Selected Human Pathogens

All the homo fermenting LAB isolates were able to inhibit indicator pathogens with inhibition zones ranging from 11-28mm (Table 5 above). *P.pentosaceus2* was however unable to inhibit *E. faecalis*. These results agree also with the findings reported by Ashenafi et al. [20] and Odugbemi et al. [21] where LAB isolates from various fermented foods displayed antimicrobial activity towards *S. aureus* and *E. coli* respectively. The findings of this study suggest that these homo-fermenting LAB isolates from kunu-zaki would be beneficial to the gastrointestinal tract of humans when kunu-zaki is consumed. This conclusion also tallies with the observations from the work of De Vries et al. [2].

3.6 Antibiotic Reaction

The results of this study show that all the homo-fermenting LAB isolates from kunu-zaki were resistant to amoxicillin, gentamycin and ciprofloxacin (Table 6 above). However, these homo-fermenters were susceptible to erythromycin with the exception of *L. plantarum 1*[26], *L. paracasei subsp paracasei*3[39] and *P. damnosus 2*[32]. Homo-fermenters that were susceptible to cotrimoxazole were *P. pentosaceus*2[27], *L. plantarum*1[33] and *P. damnosus*

The interpretive criteria used according to the manufacturer's instructions were:-

Inhibition Zone diameter (mm)
Susceptible: ≥ 10mm
Intermediate: 6-9mm
Resistant: ≤5mm
Resistant breakpoint = 5mm
Susceptible breakpoint= 10mm

4. DISCUSSION

Halting fermentation processes requires the use of high temperatures after which cooling takes place before "back-slopping" (Fig. 1). "Back slopping" is the use of a portion of freshly fermenting product to start or continue a new fermentation batch of the same product. Most traditional African fermentation processes use 'back-slopping' to ensure a continuous fermentation success. The scientific principle is that in an actively fermenting product, the population of actively fermenting organisms is quite high. The new fermentation process will therefore proceed with a less likelihood of failure if a portion of the actively fermenting product is added to it. The disadvantage of "back-slopping" however is that even though the fermentation proceeds without failure, products of inconsistent quality are often obtained from batch to batch. Kunu zaki production is heavily dependent on the method of "back-slopping". The stability of the culture at high temperatures is therefore critical. From the results obtained in this study, it would be appropriate to suggest that the development of starter cultures for kunu-zaki is an idea whose time has come.

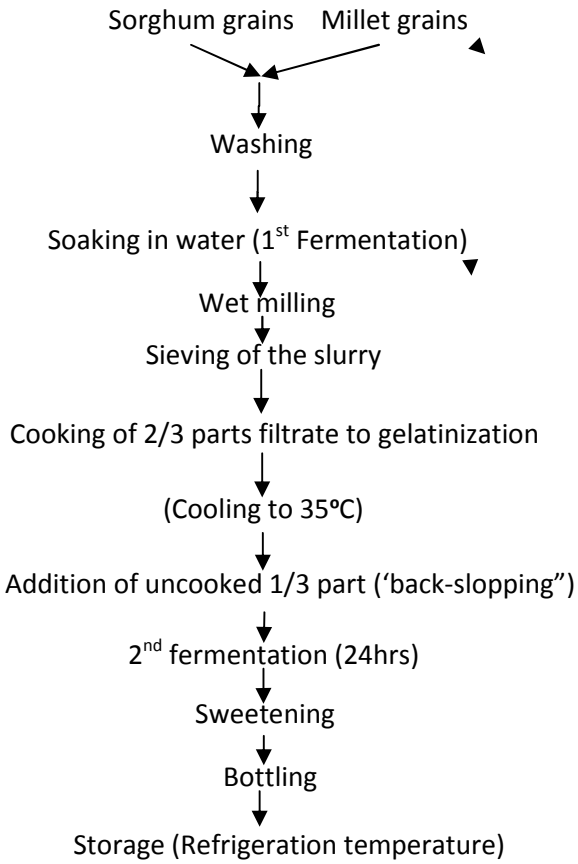

Fig. 1. Flow diagram for the traditional processing of kunu-zaki from composite grains

The introduction of health benefitting products into the market, and the use of these specific organisms as starter cultures, has constantly raised the question of antibiotic resistance and what possible interactions may occur if a consumer is taking such a product simultaneously with antibiotic therapy [22]. Plasmids are genetic elements independent of the cell chromosome. Over time, some plasmids have developed genes that make them resistant to selected antibiotics. Plasmids are easily transferred between cells, and this allows the efficient spread between bacteria of resistance to an antibiotic [7]. The number of antibiotics now available and their widespread use in prescription drugs and for general disinfection purposes seems to have increased microbial potential for the development of antibiotic resistance.

The finding of this research is significant. From the result of the adhesion study, the implication is that a regular drinker of kunu-zaki(using LAB either singly or in combination as starter culture)may likely have a high colonisation of adherent homo-fermenting starter culture LAB within his/her intestine. Furthermore, these homo-fermenters would also likely inhibit the specific pathogens tested in this study. If the consumer is on any of these antibiotic therapies, the antimicrobial activity of the LAB will likely remain unaffected by the antibiotic. However, resistance to commonly used antibiotics would raise concerns of the possibility of kunu-zaki containing antibiotic-resistant LAB bacteria passing antimicrobial resistance genes or genes that encode for virulence factors onto other resident bacteria [23].

The findings of this research however contradict the findings reported by Ketema et al. [24] that reported LAB isolated from *wakalim*, a traditional fermented beef sausage susceptible to all antibiotics tested. Strains intended for use in the food systems as starter should be carefully examined for antimicrobial susceptibility [25]. Lactobacilli are known to possess naturally a wide range of antibiotic resistance but in most cases the antibiotic resistance is not of the transmissible type as suggested by Lei and Jacobsen [22]. Of the antibiotics tested by Olukoya et al. [26], indigenous lactobacilli were resistant to cloxacillin, penicillin and streptomycin. This is comparative to the pattern revealed in our study. Also, Abdulkadir et al. [27] investigated the antimicrobial pattern of LAB isolated from fermented milk in Ethiopia. Their work noticed resistance to methicillin by all strains of LAB tested but they concluded that the LAB could not consist of reservoirs for transmissible genes of erythromycin or streptomycin resistance based on their susceptibility.

5. CONCLUSION

Homo-fermenters are usually preferred to hetero-fermenters as choice starter cultures for fermented foods since the end product of their metabolism is lactic acid only. The result of this study documents findings on the antibiotic resistance pattern of these eight homo-fermenting lactic acid bacteria present in ready to drink kunu-zaki. If these homo-fermenting strains are to be used in kunu-zaki as starter cultures, it is important that they should be further carefully examined for inability to transfer antibiotic resistance genes to food pathogens.

ACKNOWLEDGEMENT

This work was supported by a grant from the Education Trust Fund received from the Federal Ministry of Education through the Yaba College of Technology, Lagos, Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Carr FJ, Chill D, Maida N. The lactic acid bacteria: a literature survey. Crit. Rev. on Microbiol. 2002;28:281-370.
2. De Vries MC, Vaughan EE, Kleerebezem M, DeVos WM. Lactobacillus plantarum-survival, functional, and potential probiotic properties in the human intestinal tract. Int. Dairy Journal. 2006;16:1018-1028.
3. Leroy F, de Vuyst L. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trnds in Fd Sc and Technol. 2004;15:67-78.
4. Bomba A, Nemcova R, Mudronova D, Guba P. The possibilities of potentiating the efficacy of probiotics. Fd Sci and Technology. 2002;13:121-126.
5. Curragh HJ, Collins MA. High levels of spontaneous drug resistance in Lactobacillus. J. Appl. Bacteriology. 1992;73:31-36.
6. Catalouk O, Gogebakan B. Presence of drug resistance in Lactobacilli of dairy & human origin in Turkey. FEMS Microbiol Lett. 2004;236:7-14.

7. Perreten V, Schwarz F, Cresta L, Boeglin M, Dasen G, Teuber M. Antibiotic resistance spread in food. Nature (London). 1997;389:801-802.
8. Tynkkynen S, Singh KV, Varmanen P. Vancomycin resistance factor of Lb. rhamnosus GG in relation to enterococcal vancomycin resistance (van) genes. Int. J. Fd Microbiol. 1998;41:195-204.
9. Gfeller KY, Roth M, Meile L, Teuber M. Sequence and genetic organization of the 19.3-kb erythromycin and dalfopristin-resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1.Plasmid. 2003;50:190-201.
10. Efuewevwere BJO, Akoma O. The microbiology of kunu zaki: a cereal beverage from Northern Nigeria during the fermentation production process. World J. Microbial Biotechnol. 1995;11:491-493.
11. Elmahmood AM, Doughari JA. Microbial quality assessment of kunu-zaki beverage sold in Girer town of Adamawa State, Nigeria. Afr. J. Fd Sc. 2007;1:11-15.
12. Adeyemi A, Umar S. Effects of methods of manufacture on the quality characteristics of kunu-zaki; a millet based beverage. Nig Fd. J. 1994;12:33-40.
13. Bulut C. Isolation and molecular characterisation of lactic acid bacteria from cheese. IYTE Thesis of MSc; 2003.
14. Rojo-Bezares B, Senz Y, Poeta P, Zara Zaga M, Ruliz-Larrea C. Assessment of Antibiotic susceptibility within lactic acid bacteria strains isolated from wine. Int. J. Fd Microbiol. 2006;111:234-240.
15. Mardh PA, Westron L. Infection and Immunity. 1976;13:661. [PUBMED].
16. Otero MC, Nader-Macias ME. Lactobacillus adhesion to epithelial cells from bovine vagina. Comm. Curr. Res. & Educ. Topics & Trnds in Appl. Microbiology A. Mendez-Vilas (Ed). 2007;749-757.
17. Amusa NA, Ashaye OA. Effect of processing on nutritional, microbiological and sensory properties of kunu-zaki. Pak. J. Nutr. 2009;8(3):288-292.
18. Odunfa SA. African fermented foods In: Microbiology of Fermented Foods Vol2 (First Edition). London: Wood, B.J.B (Ed) Elsevier Applied Science. 1985;155-191.
19. Collins JK, Thornton G, Sullivan GO. Selection of probiotic strains for human applications. Int Dairy J. 1998;487-490.
20. Ashenafi MG Tadasse, E Eden. Assessment of the antimicrobial activity of lactic acid bacteria isolated from Borde & shamita, traditional Ethiopian fermented beverages, on some food-borne pathogens & effect of growth medium on the inhibitory activity. Int. J. of Fd Safety. 2011;(5):13-20.
21. Odugbemi T, Odujinrin OMT, Akitoye CO, Oyerinde JPO, Esumeh FI. Study on the pH of Ogi, Nigerian fermented weaning food & its effect on enteropathogenic *E. coli, S. typhi* & *S. paratyphii*. J. Trop. Med. & Hyg. 1991;94:219-223.
22. Mathur S, Singh R. Antibiotic resistance in food lactic acid bacteria. A review. Int J. Fd Microbiol. 2005;105:281-295.
23. Lei V, Jakobsen M. Microbiological characterisation and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. J. App. Microbiol. 2004;96:384-397.
24. Ketema B, Tetemke M, Mogessie A. Antibiotic Susceptibility Patterns of LAB isolated from wakalim, a traditional Ethiopian Fermented Beef Sausage. J. Fd. Safety. 2010;30:213-223.
25. Teuber M, Meile L, Schwarz F. Acquired antibiotic resistance in lactic acid bacteria from food. Anton. Leewen. 1999;76:115-137.

26. Olukoya DK, Ebigwei SI, Adebawo OO, Osiyemi FO. Plasmid profiles and antibiotic susceptibility patterns of Lactobacillus isolated from fermented foods in Nigeria. Fd Microbiol. 1993;10:299-285.

27. Abdulkadir B, Tsige K, Ketema B. Antimicrobial susceptibility pattern of lactic acid bacteria isolated from Ergo, a traditional Ethiopian fermented milk, Jimma, South West Ethiopia. Ethiop. J. Educ & Sc. 2011;7(1):9-17.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=605&id=8&aid=5413