Original Research Article

Antimicrobial Activity of Lactic Acid Bacteria from Kunun-Zaki on Selected Clinical Pathogens

ABSTRACT

Aims: To investigate the anti-microbial activity of lactic acid bacterial isolates from locally fermented Kunun-zaki beverage.
Study design: The study was designed to study the anti-microbial effect of lactic acid bacterial isolates obtained from Kunun-zaki beverage.
Place and Duration of Study: Department of Applied Microbiology Enugu State University of Science and Technology, Agbani, June 2009 and January 2010.
Methodology: Fresh Kunun drink was prepared from millet following standard modified procedures. Isolation of lactic acid bacteria from the beverage sample was carried out using MRs agar medium. Morphological and biochemical characterizations were perform to identify the organisms. Finally, the isolated bacterial strains were tested for their ability to inhibit the growths of clinical pathogens – *Bacillus antracis, Clostridum preferengenes, Escherichia coli, Streptococcus faecalis, Micrococcus luteus, Klesiella pneumonia, Pseudomonas acnignosa, Bacillus cereus, Bacillus substilis* and *Staphylococcus aureus*. **Results:** Morphological and biochemical characteristics revealed the presence of lactic acid bacteria (LAB) from Kunun-zaki drink which were identified as *Lactobacillus casei* and *Lactobacillus brevis*. The antimicrobial activity of the isolates (*L. casei* and *L. brevis*) against the infectious strains - *Bacillus antracis, Clostridum preferengenes, Escherichia coli, Streptococcus faecalis, Micrococcus luteus, Klesiella pneumonia, Pseudomonas acnignosa, Bacillus cereus, Bacillus substilis* and *Staphylococcus aureus* recorded 90 % inhibition against the pathogens. *Micrococcus luteus* was resistant to all forms of LAB treatments.
Conclusion: The study concludes that *Lactobacillus brevis* and *Lactobacillus casei* from kunun-zaki drink possess strong antimicrobial activity against a wide range of clinical pathogens. And since lactic acid bacteria are known to possess many health benefits, some of the therapeutic effects reported from the consumption of the beverage may be attributed to these organisms.

Keywords: Antimicrobial, Lactic acid bacteria, Kunun-zaki, Clinical pathogens

1.0 INTRODUCTION – make the references in square bracket [ ] not in this ( )

Indigenous fermented food prepared from major cereals are common in many parts of Africa. Some are used as beverages and breakfast or snack foods while a few others are consumed as staples and weaning foods. Kunun-zaki is a traditionally fermented non-alcoholic beverage widely consumed in Northern Nigeria. It is produced either from millet (*Pennisetum typhoideum*) or sorghum (*Sorghum bicolor*). It is believed to have immense nutritional and medicinal importance to its numerous consumers. Kunun-zaki has been reported to contain carbohydrates, B-vitamins and numerous minerals but low in proteins (1). The increase in acidity (i.e. lactic acid) of Kunun-zaki during production has been attributed

to the dominance of *Lactobacillus leichmanni and Lactobacillus fermentum* during the fermentation process (2).

Lactic acid bacteria are technically called thermophiles due to their ability to grow well above 38^{o}C. LAB is among the probiotic bacteria that produce substances that inhibit the growth of pathogenic, non-pathogenic and spoilage organisms in fermented foods and beverages (3). The antimicrobial activity of LAB maybe due to their ability to produce toxic substances such as organic acid, diacetyl, hydrogen peroxide and bacteriocin. The organic acid includes lactic, acetic and propionic acid. Hydrogen peroxide is oxidative and bactericidal. Bacteriocin is a short peptide or protein produced by lactic acid bacteria with a bactericidal property. For example, Nisin is recognized as bactericidal to Gram positive bacteria preventing the outgrowth of *Bacillus* or *Clostridum* spores (4).

The pH value of lactic acid fermented foods is reported to be less than 4, which is also sufficient to suppress the growth of most food borne pathogens. Most pathogens have critical pH limits beyond which they cannot grow. However, the extent to which pathogens are inhibited by low pH value is further affected by the surrounding temperature, undissociated acid and buffer capacity of the food. The undissociated acid may diffuse into the bacterial cell thus reducing the intra-cellular pH and slowing down metabolic activities. However, in order to produce a sufficient acidic medium to inhibit the growth of bacterial pathogens, relatively large numbers of (up to 10^9 cfu/1ml) of lactic acid bacteria must be present (4).

2.0 EXPERIMENTAL DETAILS (make as Materials and Methods)

2.1 Laboratory Preparation of Kunun-zaki

The millet sample (500 g) was cleaned and steeped in 100 ml of tap water (1:2 w/v) for 24 h. The water was decanted and the grains with the spices were washed with water and milled with 2 volume of tap water. The resulting paste (800 g) was divided into two equal parts (usually 1:3w/v). One part was sieved, cooked with two volumes of boiled water and allowed to cool. The other part was sieved and mixed with the cooked portion. The mixture was left for 10 h to ferment and sugar was added (5).

2.2 Preparation of Media

The MRs agar medium was used according to the manufacturer's instruction **(give the company name).** About 62.2 g of MRs agar was dissolved in 1 L of distilled water and heated to dissolve. The solution was sterilized by autoclaving at 121^{o}C for 15 min and used for isolation of the lactic acid bacteria. Nutrient agar medium **(ref.)** was used to maintain the reference organisms collected from the Microbiology Department of Obafemi Awolowo, Ile –Ife.

2.3 Isolation of Bacteria from Kunun-zaki

2.3.1 Serial dilution

About 1 ml of kunun-zaki was dispensed aseptically into a sterile test tube to which 8 ml of 0.1 % sterile peptone water had been previously added. The mixture was shaken to homogenize and a dilution factor of 10^{-1} was obtained. Then 1 ml of this dilution (10^{-1}) was pipetted and dispensed aseptically into another sterile test tube containing 8 ml of sterile diluents (0.1 % sterile peptone water) this makes a mixture of one in hundred dilution i.e. 10:2. The process was repeated until a dilution of seven fold was obtained.

2.3.2 Inoculation

The media was inoculated with inoculums and incubated to dilution factors of 10^{-3} and 10^{-5} and used for the isolation. Exactly 0.1 ml each of the two dilutions was transferred into clean and sterile petri dish in duplicates. This was followed by the addition of 10 ml of the molten and sterile lactic agar medium. The plate was rotated carefully to allow even distribution of the inoculum within the medium. This was then allowed to set and solidify. The plates were incubated at 30 ^{o}C anaerobically for 24-48 h and observed for growth. The isolates were sub-cultured until pure culture was obtained. The pure culture was then used for morphological and cultural characterization of the isolates. The pure isolate was again cultured in the MRs agar slant as stock culture for subsequent experiment (6).

2.3.3 Preparation of Antimicrobial Disk

The antimicrobial disc was prepared by punching out discs of 6 mm diameter on Whattman filter paper. Each disc was labeled with number corresponding to the number assigned to each isolate for easy identification. The disc was placed in a petri dish sealed and sterilized in the autoclave at 121 ^{o}C for 15 min. After sterilization, they were then dipped into the culture broth and incubated at 31 ^{o}C for 24 h (7).

2.4 Impregnation of Antimicrobial Discs into the Culture Medium

The prepared nutrient agar medium was aseptically poured into clean and sterile petri dish. The agar plate was seeded with the corresponding organism by placing a loopful of inoculums at the center of the plates and spreading all over the agar surface with the sterile cotton swab to enable uniform lawn of growth (7). This was left to stand for 25 minutes then, antimicrobial disc was placed on the plates. The plate was kept at 4 ^{o}C for 3-4 hours to allow for proper diffusion and later incubated at 7^{o}C for 14-16 h. After incubation, the plate was observed carefully. The morphology of each colony was examined and recorded on the basis of size, form, pigmentation, margin, elevation and opacity for evaluation of microscopic character, pure colony of each isolates was picked and Gram staining was performed. The size, shape, arrangement and Gram reaction properties of isolates were carefully observed.

2. 5 Morphological Characteristics of Isolates

2.5.1 Gram Staining Techniques

Gram staining was carried out on the isolates. A smear of the culture was made on a clean grease free slide labeled with each isolate code and heat fixed to dry. The smear was then stained with crystal violet for 60 seconds after which it was rinsed in water. Few drops of Lugol's iodine solution (grams iodine) was added and allowed for 60 seconds. The smear was then decolourized with 95% ethanol for 30 seconds and immediately rinsed with tap water. The slide was counter stained with Carbon Fuchsin for 1 min and rinsed with water and then dried with Whatman filter paper. Gram positive cells are purple while Gram negative cells are red **(ref.).**

2.6 Biochemical Characteristic of the Isolates

2. 6.1 Catalase Test

A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of microorganism from 24-hour pure culture was placed onto the microscopic slide. 3% H_2O_2 solution was added to each of the slides and a portion of the bacterial colony was mixed with it. Production of bubble indicated the presence of catalase enzyme in the bacteria (8).

2.6.2 Oxidase Test

Aliquot of the colony was picked with a tooth pick and rubbed on a strip of a filter paper impregnated with oxidase reagent (1% aqueous solution of N'N'N'N'12 tetramethyl-p-phenylenediaminedihydrochloride). Oxidase test indicates positive by the presence of dark purple color within 10 seconds (9).

2.6.3 Motility Indole Urea (MIU) Test

Following incubation for 18-24 h at 37°C, the colony in tube was observed for the presence of motile organisms. For indole production, 0.1 ml of chloroform was first added to each test tube and allowed for 15 min. Then 1ml of Kovac's reagent was added and then examined for red color development within 10 minutes as an indication of indole production (10).

2.6.4 Methyl Red Test

Methyl red test was performed to determine the ability of the bacteria to oxidize glucose with the production of high concentration of acidic end products. Glucose phosphate broth was inoculated with the isolates and incubated for 2-5 days at 37˚C. Then 5 drops of methyl red indicator (0.2%) was added and carefully observed for immediate development of a red color which indicates positive test for acidic products (10).

2.6.5 Voges- Proskauer Test

The isolates were aseptically inoculated into glucose phosphate broth tubes; one of the tubes was left un-inoculated to serve as control. Both tubes (inoculated and un-inoculated) were incubated for

48 h at 37˚C after which 3 drops of Barrits reagent was added. The test was observed for pink coloration for positive Voges- Proskauer test (10).

2.6.6 Starch Hydrolysis Test

Starch hydrolysis tests the ability of the microorganism to utilize starch as carbon and energy source for growth through the use of α-amylase. The starch agar plate was inoculated and incubated at 37°C for 48 hours. Iodine reagent was added to surge the growth. Iodine reagent was added after incubation to flood the surface of the plate using a dropper pipette. Changes in the plate was monitored. The starch in the plate was changed to blue-brown by the iodine reagent. Zones where starch has been hydrolyzed by bacterial growth displayed clear halos in the midst of the dark plate, demonstrating a positive α-amylase or starch hydrolysis test. Plates containing bacteria without α-amylase activity was uniformly dark (11).

2.6.7 Sugar Fermentation Test

Sugar fermentation was used to test the ability of the bacteria to ferment sugar such as lactose (disaccharide), sucrose (disaccharide), Sorbose (Monosaccharide) and Mannitol (an alcoholic sugar). The based medium of peptone substrate (0.5-1%) was prepared and 1% Andrade's indicators was added. The medium was dispensed into 5 sterile Durham tubes and autoclave. The sterile medium was inoculated with broth culture and incubated at 35^{o}C for 24-48 h. The culture tubes were observed for gas and acid productions (12).

2. 7 Anti-Bacterial activity of the isolates against Clinical Pathogens

The bacterial strains isolated from Kunun-zaki were studied to determine their ability to kill pathogenic bacteria such *as Klebsiella pneumoniae, Micrococcus luteus, Clostridium perfringes, Bacillus anthracis, Streptococcus Faecalis, Bacillus Cereus, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherischia coli*. The test organism was placed on Muller-Hinton (MH) agar plates. Each bacterial isolate was then inoculated as small spot with appropriate labeling for easy identification. The plates were incubated at 37 ^{o}C for 24 h. Bacteria which can kill or produce chemicals that inhibit bacterial growth exhibited clear transparent zones around the inoculated spot of inoculation. After 24 h of incubation, the diameter of zone of inhibition was determined and recorded (12).

3.0 RESULTS AND DISCUSSION

Bacterial colonies with different morphology including differences in size, shape, colour and elevation were observed. Morphological characterization revealed the isolates to be *coccibacilli* rod. The colonies had raised elevations however, only a colony (ID) had dome shape. The diameter 0.1-0.2 mm was observed in all. All the isolates were translucent with color ranging from cream to deep green. In order to characterize the isolates, gram staining and biochemical tests were performed. Result showed

that all the isolates were gram positive. In microscopic analysis, the isolated bacteria were all cylindrical, transparent and with raised or dome elevations (Table 1).

Table 1: Morphological Characterization of the Isolates

Isolate No	Color	Description of Colony	Margin	Gram Staining
1A	Cream	Cylindrical, large (0.1-0.2 mm), transparent, raised elevation	Entire	+
1B	Cream	Cylindrical, large (0.1-0.2 mm), transparent, raised elevation	Entire	+
1C	Deep green	Cylindrical, punctiform, transparent, raised elevation	Entire	+
1D	Cream	Cylindrical, large (0.2 mm), transparent, dome elevation	Undulate	+
1E	Deep green	Cylindrical, punctiform, transparent, raised elevation	Entire	+

3.1 Biochemical Characterization

The biochemical characterization showed that, the colony characteristics of the isolates were different from each other. Three isolates 1C, 1D and 1E had acid and gas production; while the other two isolates (1A and 1B) had acid production only. None of the isolates utilized xylose for energy source. 1A and 1B had acid production for sorbose utilization whereas 1C, 1D and 1E showed no change (Table 2).

The isolates had a cocci shape, gram positive, negative oxidase and catalase tests, 1A-1B did not hydrolyze starch however 1C-1E metabolized the starch. SIM reaction was negative in all. Methyl red (Mr) was positive while Vp was negative. There was no fermentation with xylose. There was a utilization of sorbose, mannitol, sucrose, lactose and even non-sugar sodium chloride. Salt tolerance experiments mainly test the tolerance ability of the organism to NaCl and other salts, and determine the optimum concentration for growth. From these biochemical tests, the possible organisms were identified to be *Lactobacillus casei* and *Lactobacillus brewis.*

Table 2: Biochemical characterization of the *lactobacilli* isolates

Isolate No	Shape	Oxidase test	Catalase test	Starch hydrolysis	Sim reaction	Mr test	Vp test	Xylose	Sorbose	Mannitol	Sucrose	Lactose	Growth in 4% NaCl	Possible organism
1A	***cocobacilli***	**-**	**-**	**-**	**-**	**+**	**-**	**NC**	**Y**	**Y**	**NC**	**NC**	**+**	***L Casei***
1B	***cocobacilli***	**-**	**-**	**-**	**-**	**+**	**-**	**NC**	**Y**	**Y**	**NC**	**NC**	**+**	***L Casei***
1C	***cocobacilli***	**-**	**-**	**+**	**-**	**+**	**-**	**NC**	**NC**	**YG**	**YG**	**YG**	**+**	***L Brewis***
1D	***cocobacilli***	**-**	**-**	**+**	**-**	**+**	**-**	**NC**	**NC**	**YG**	**YG**	**YG**	**+**	***L Brewis***

1E	***cocobacilli***	**-**	**-**	**+**	**-**	**+**	**-**	**NC**	**NC**	**YG**	**YG**	**YG**	**+**	***L Brewis***

Key: (+) = positive reaction; (**-**) **=** negative reaction; (YG) = acid and gas production; (Y) **=** acid production only; (nc) = no change; Mr = methyl red; Vp = Voges- Proskauer; NaCl = sodium chloride

Table 3: Zone of inhibition (mm) for *L. casei, L. brevi* and mixed culture against other strains

S/NO	Microorganism	Zone of inhibition (mm) Due to *Lactobacillus casei*	Zone of inhibition (mm) Due to *Lactobacillus brevis*	Zone of inhibition (mm) Due to Mixed Culture
1	*Klebsiella pneumoniae*	13.5±0	27.0±0	24.0±0.5
2	*Micrococcus luteus*	0±0	0±0	0±0
3	*Clostridium perfringes*	9.5±0.5	21.0±1.5	24.0±0.5
4	*Bacillus anthracis*	10.5±0.5	16.0±0.5	13.0±0.5
5	*Streptococcus Faecalis*	10.0±0	18.0±0.5	13.5±0.5
6	*Bacillus Cereus*	23.0±1.5	23.0±1.5	23.0±0
7	*Bacillus subtilis*	10.5±1.5	21.0±0.5	20.0±0
8	*Pseudomonas aeruginosa*	11.0±0.5	20.5±1.0	25.0±1.0
9	*Staphylococcus aureus*	12.0±1..0	19.0±0	12.5±0
10	*Escherischia coli*	20.5±1.0	24.0±0.5	15.5±0
11	Control	6.0±0	6.0±0	6.0±0

From indication, the mixed culture tend to have stronger anti-bacterial activity against the tested strains. This was followed by *Lactobacillus brevis* while the least activity was obtained with *Lactobacillus casei.* All the test strains were inhibited except *micrococcus luteus* which showed absolute resistance to the lactic acid bacteria both individually and in mixed form.

Kunun-zaki is a good source of essential nutrients for humans and microbes. Its nutritional compositions include carbohydrates, B-vitamins, minerals and low protein content; making it an ideal medium for microbial growth. The acidity (i.e. lactic acid) of Kunun-zaki is attributed to the presence of *Lactobacillus leichmanni* and *Lactobacillus fermentum* during the fermentation process (13).

In the continued search for safe and effective broad spectrum antibiotics for the management of infectious diseases resulting from drug resistance, this study investigated the antimicrobial activity of lactic acid bacteria in kunun-zaki beverage against selected clinical pathogens. The indiscriminate use of modern antibiotics in the treatment of infectious diseases is beginning to fail and in most cases, leads to increase in antibiotic resistance (13, 14). This study revealed the possibility of sourcing potent antimicrobial agents from kunun-zaki as the isolates elicited potent antibacterial activity against the clinical pathogens.

The fermentation reaction by the isolates of LAB in basic sugars were positive for sorbose and mannitol but partial in the utilizations of sucrose and lactose. Moreover, MR reaction and growth in NaCl were also positive for all the isolates. All the biochemical tests result of twelve isolated organisms were

observed and the entire organisms were identified as *Lactobacillus brevis* and *Lactobacillus casei*. The organisms were taken for antibiotic test against clinical pathogens.

In respect to such antimicrobial susceptibility testing, almost all of the clinical pathogens were susceptible to *Lactobacillus brevis***,** *Lactobacillus casei* and the mixed culture. Only *Micrococcus luteus* was insensitive to *Lactobacillus brevis* and *Lactobacillus casei* treatments. Such resistance may have evolved as a result of the ability of *Micrococcus luteus* to produce defensive molecules or enzymes that neutralized the inhibitory effects of the LAB isolates. Further studies are required to understand the molecular basis of the development of antibiotic resistance of these strains. *Clostridium perfringes* was intermediate recalcitrant to *Lactobacillus casei* but highly sensitive to *Lactobacillus brevis* and mixed culture. *Staphylococcus* spp. isolates have also been reported to be intermediate recalcitrant to penicillin and oxacillin treatment (12). In contrast, *Klebsiella pneumoniae* was the most sensitive organism to *Lactobacillus brevis* treatment, followed by *Pseudomonas aeruginosa* treated with the mixed culture of *Lactobacillus brevis* and *Lactobacillus casei*. The antimicrobial activity demonstrated by the isolates covered a wide range of possible infections and offers an alternative therapeutic option for the treatment of infections caused by these bacteria.

Finally, this study has uncovered the presence of lactic acid bacteria *Lactobacillus brevis* and *Lactobacillus casei* in locally fermented kunun-zaki beverage. Their presence in this beverage is associated with many health benefits including protection from enteric pathogens; stimulation of immune system and maintenance of gastro-intestinal microflora balance as well as mediating the release of vital nutrient molecules during the fermentation process.

4.0 CONCLUSION

The study concludes that *Lactobacillus brevis* and *Lactobacillus casei* from kunun-zaki drink possess strong antimicrobial activity against a wide range of clinical pathogens. And since lactic acid bacteria are known to possess many health benefits, some of the therapeutic effects reported from the consumption of the beverage may be attributed to these organisms.

CONFLICT OF INTERESTS

The authors have no conflicts of interests.

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