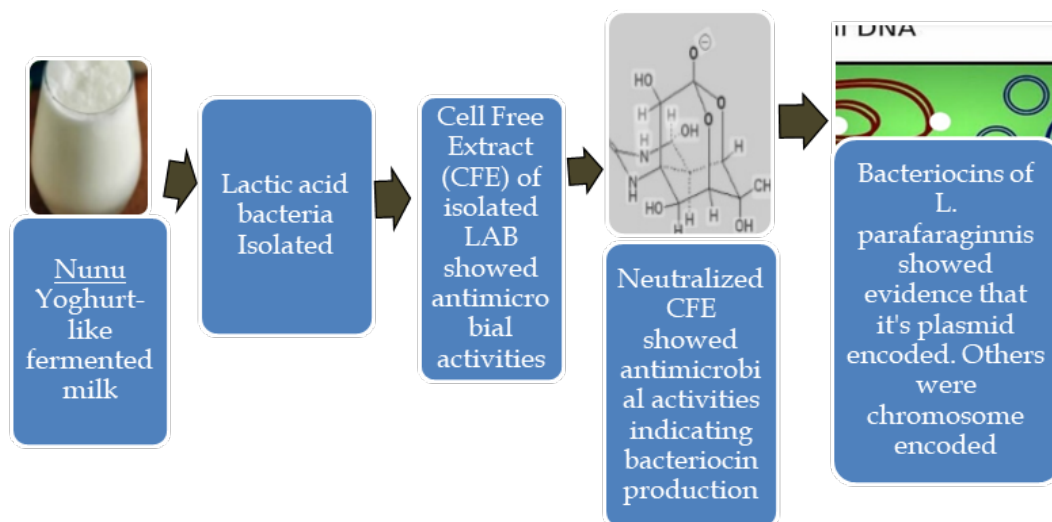


RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITIES AND BACTERIOCINS OF LACTIC ACID BACTERIA ISOLATED FROM NIGERIA INDIGENOUS FERMENTED COWMILK “NUNU”

B.K. Saliu*, H. Abdulkadir, V.O. Dadzie, A. A. Alabi, R.I. Salami, and A.E. Ajewole



Highlights

- *Lactobacillus parafarraginnis* OCLP1, *L. fermentum* CSL58 and *L. buchneri* MZE12 isolated from ‘nunu’ exhibited antimicrobial activity.
- *Bacillus anthracis* CHF-2011-32b, *B. cereus* DHM5-1, *Providencia vermicola* KSVDL-09-71600, *P. rettgeri* SIRT3, *P. stuartii* SILT11 were the test isolates.
- The cell-free extract of Lactic acid bacteria, especially *L. parafarraginnis* OCLP1, demonstrated a wide spectrum of activity.
- Bacteriocins of *L. parafarraginnis* OCLP1 was plasmid-encoded, while the rest were encoded on chromosome.

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ANTIMICROBIAL ACTIVITIES AND BACTERIOCINS OF LACTIC ACID BACTERIA ISOLATED FROM NIGERIA INDIGENOUS FERMENTED COWMILK “NUNU”

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Abstract: Nunu is a nutritious fermented yoghurt-like milk beverage that can serve as a source of lactic acid bacteria (LAB) with antimicrobial and bacteriocin potentials. LAB were isolated from nunu samples using MRS agar and screened for antimicrobial activity against test organisms. Three LAB, namely *Lactobacillus parafarraginis* OCLP1, *L. fermentum* CSL58 and *L. buchneri* MZE12 out of a total of 14 isolates produced antimicrobial activity against *Bacillus anthracis* CHF-2011-32b, *B. cereus* DHM5-1, *Providencia vermicola* KSVDL-09-71600, *P. rettgeri* SIRT3, *P. stuartii* SILT11 and other test organisms. Antimicrobial factors were assayed in LAB cell-free extracts (CFE), neutralized CFE, and ammonium sulphate precipitants of the CFE, and *Lactobacillus parafarraginis* OCLP1 demonstrated the highest activity with zones of inhibition of 20.33±3.51 mm, 13.33±1.09 mm and 25.33±2.52 mm, respectively. The CFE of the LAB especially *L. parafarraginis* OCLP1 demonstrated wide activity spectra by inhibiting both the Gram-positive and Gram-negative bacteria tested. Result of plasmid curing of the LAB indicated that the bacteriocins of *L. parafarraginis* OCLP1 was plasmid-encoded while the others have their gene clusters on the chromosome. This study concluded that bacteriocin-producing LAB can be found in “nunu” and recommend the use of the isolated LAB as starters in fermented food production after ensuring their safety through toxicological studies.

Keywords: Fermented milk; Nunu; *Lactobacillus*; Antimicrobials; Bacteriocins

INTRODUCTION

Nunu is a yoghurt-like traditional milk product consumed in many West African countries including Nigeria. It is rich in protein, essential amino acids, phosphorous, calcium as well as vitamins A, C, E and B complex (Nebedum & Obiakor, 2007). Nunu is produced through spontaneous and uncontrolled fermentation of cowmilk. The traditional fermentation is often initiated by adding “nunu” from previous ferments. Fermenting organisms attached to the calabash container may also initiate fermentation. The fermented milk is churned to separate milk fat i.e., the buttermilk known as “maishanu” in Nigeria, and the watery part is the “nunu”.

Many microorganisms including bacteria and yeasts have been isolated from “nunu”. Notable are the lactic acid bacteria which acidify the milk and confer it the characteristic and desirable sour taste. In addition, LAB exert preservative effect on the milk, due to production of

organic acids (Daeschel, 1989), hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins (Cintas et al., 2001).

Bacteriocins are a heterogeneous group of bioactive, ribosomal synthesized, bacterial peptides or proteins, that display antimicrobial activity against other bacteria, both closely related and non-related (Karpinski & Szkaradkiewicz, 2016). Through the inhibition of spoilage and pathogenic bacteria, bacteriocins have found applications in food preservation, ultimately contributing to food safety and security (Gundogan, 2014), and in animal feeds and pharmaceuticals. Bacteriocins bind to specific receptors on bacterial cells and form pores in the membrane, causing cell permeability, and leading to the breakdown of DNA, inhibition of protein and breakage of cellular contents i.e., ions and ATP, eventually resulting in cell death (Preciado et al., 2016). Bacteriocins are either chromosome or plasmid encoded, or both. *Escherichia coli* for instance, encodes its colicins exclusively on plasmid replicons (Pugsley et al., 1987) while pyocins of *Pseudomonas aeruginosa*, a close relative of colicin and some other bacteriocins are found exclusively on the chromosome (Sano et al., 1990). The bacteriocins of *Serratia marcescens*, which are also close to the colicins, are found on both plasmids and chromosomes (Guasch et al. 1995). The clusters for the lantibiotics nisin from Gram-positive bacteria are most often encoded on plasmids but are occasionally found on the chromosome especially on transposons (Dodd et al., 1990)

Nunu has the potential to serve as a vehicle for administering bacteriocin-producing LAB with all the derivable benefits to human health particularly in the gastrointestinal tract, in addition to ensuring food security through preservation. However, these LAB may not be present in all retailed “nunu” due to the spontaneous home-based fermentation, whereby there is no control on the type of fermenting organism used. With a view to developing organisms with properties including bacteriocin production as starters for milk fermentation, the present study investigated bacteriocin-producing LAB in “nunu” and determined whether the bacteriocin was chromosome or plasmid encoded.

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MATERIALS AND METHODS

Isolation of Organisms from Nunu

Lactic acid bacteria were isolated from “nunu” samples previously collected from retailers in Ilorin, Kwara State, using de Mann Rogosa and Sharpe (MRS) Agar. Other media were used to isolate organisms from the sample for use as test organisms. MacConkey Agar (MA) was used for fecal coliforms, Nutrient Agar (NA) as general medium, Salmonella-Shigella Agar (SSA) as specified, and Mannitol Salt Agar (MSA) for *Staphylococcus aureus*. All the isolates were characterized by their colonial morphology on agar medium, microscopic appearance following Gram and spore staining procedures and biochemical tests (Fawole & Oso, 2007), while some with notable results were identified after molecular characterization. Known organisms obtained from the Microbiology and Parasitology Department of the University of Ilorin Teaching Hospital were also used as test organisms.

The molecular characterization followed the method described by Lear et al. (2018), QIAamp DNA Mini Kit (250) cat No. 51306 was used to extract genomic DNA and the 16SrRNA genes were amplified using the 16SF (5'AGAGTTTGATCCTGGCTCAG 3') and 16SR (5'TACCTTGTTACGACTT 3') primers. The PCR product was purified from contaminants by electro-elution of the gel slice containing the excised desired fragments with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 300 µL of nuclease free water in order to enhance the purification of the PCR products. Sequencing was performed using automated sequencer (ABI PRISM 310, Applied Biosystems, USA). Translated nucleotide sequences were analysed for similarities using BLASTN tool (www.ncbi.nlm.nih.gov:80/BLASTN/). ClustalW Multiple Sequence Alignment was constructed using BioEdit v7.2.5 for Windows.

Screening of Isolates for Antagonistic Activities

All the organisms isolated on MRS Agar were screened for the ability to inhibit those isolated on the other media, and test organisms (*Yersinia enterocolitica*, *Salmonella enteritidis*, *Candida albicans*, *Serratia liquifacien*, *Citrobacter freundii* and *Staphylococcus aureus*) obtained from the Microbiology and Parasitology Department of the University of Ilorin Teaching Hospital. Pure colonies of the presumed LAB isolates were cultured in MRS broth for 48 hours and centrifuged at 6000 x g, 4 °C for 15 minutes to obtain cell-free extracts (CFE). The supernatants were collected and used to challenge test organisms by agar-well dilution assay as described by Cardirci & Citak (2005). Plates were incubated at 37 °C and observed daily for growth and to detect inhibition zones. The diameter of the zones of inhibition were measured in five planes and mean used to represent antagonistic activities. Organisms that showed antagonistic activity with a zone of inhibition ≥ 5.00 mm, and the corresponding susceptible test organisms were used in the downstream study while the others were discarded.

Determination of the Antimicrobial Principle of Lactic Acid Bacteria Isolated from Nunu

The CFE of individual LAB isolates were neutralized to pH 7.0 using 2M NaOH and used to challenge test organisms by agar well-diffusion method to eliminate acid as the sole antimicrobial principle. Protein precipitation of the neutralized CFE was done using ammonium sulphate $\{(NH_4)_2SO_4\}$ (80% w/v) (Bollag & Edelstein, 1991). The pellets obtained after centrifugation at 6000 x g, 4 °C for 30 minutes were re-suspended in 2 ml of potassium phosphate buffer (pH 6.8) and used to challenge test organisms as a means of detecting bacteriocin.

Spectrum of Activity of the Cell-free Extracts of Lactic Acid Bacteria Isolated from Nunu

The ability of the LAB isolates to inhibit *Yersinia enterocolitica*, *Salmonella enteritidis*, *Candida albicans*, *Serratia liquifacien*, *Citrobacter freundii* and *Staphylococcus aureus* all of which are common pathogenic organisms along with those isolated in this study, was determined. The pathogens were challenged with the CFE of the LAB isolates using the agar-well diffusion method. Diameter of inhibition zones were measured after 24 hours of incubation at 37 °C.

Determination of the Location of the Bacteriocin Gene Cluster of the Lactic Acid Bacteria Isolates

Plasmid curing was employed to determine whether the bacteriocin of the LAB isolate was plasmid encoded or otherwise, using sodium dodecyl sulphate (SDS) treatment according to Kouakou et al. (2010). Briefly, 0.1 ml of overnight MRS broth culture of the LAB isolate was inoculated into a fresh MRS broth containing 1% SDS and incubated at 42 °C overnight. The culture was centrifuged at 4000 x g for 5 min. The resulting pellet was resuspended in 5.0 ml saline, diluted to 10^{-5} , inoculated on MRS agar plates by spreading, and incubated overnight at 37 °C. The overnight agar culture was overlaid with 3mL of soft (0.75 %) agar medium already seeded with 30 µl broth culture of the test organism at 10^6 CFU ml⁻¹. The plates were again incubated overnight at 37 °C and observed for clear zones around colonies of LAB isolates. Absence of clear zones indicate that there was no antimicrobial activity and was used to imply that the bacteriocin was plasmid encoded.

Data Analysis

All data are means of triplicate measurements ± standard deviation (SD). Measures of significance were obtained through ANOVA (Analysis of variance) using SAS 9.0 software.

RESULTS AND DISCUSSION

Organisms

Three out of fourteen organisms isolated on MRS agar produced inhibition zones greater than 5.00 mm against the test organisms which included those isolated on Nutrient Agar, MacConkey, and Mannitol Salt Agar, and some clinical isolates previously obtained from the University of Ilorin Teaching Hospital (Table 1).

Table 1: Inhibition of test organisms by Isolated Lactic Acid Bacteria

LAB Isolates	Inhibition of test organisms										
	B1	B2	B3	B4	B5	YE	SE	CA	SL	CF	SA
L2	+	+	+	+	+	+	+	+	+	+	+
L11	+	+	+	-	+	-	+	-	-	-	-
L13	+	-	+	-	+	+	+	-	-	+	-

“+” sign: denotes positive “-” sign: denotes negative, L2, L11 and L13 isolates on de Mann Rogossa Sharpe Agar;

B1 – B5 other isolates; Y.E: *Yersinia enterocolitica*; S.E: *Salmonella enteritis*; CA: *Candida albicans*; SL: *Serratia liquifacien*; CF: *Citrobacter freundii*; and SA: *Staphylococcus aureus*

The morphological and biochemical characteristics of the bacterial isolates are presented on table 2 while table 3 and figure 1 show their identity based on molecular characteristics. All the three isolates from MRS agar (designated L2, L11 and L13) were Gram-positive, motility, catalase and oxidase-negative, and non-sporulating. All produced acid from the sugars used except L2 which did not utilize sucrose. The three isolates did not grow at 6.5% NaCl and apart from L11, the other two were thermophilic as they grew well at 45 °C. These characters were used to tentatively classify the isolates as lactic acid bacteria. Isolate L2 was identified as *Lactobacillus parafarraginis* OCLP1, L11 as *Lactobacillus fermentum* CSL58 and L13 as *Lactobacillus buchneri* MZE 12 following the amplification and sequencing of the organisms’ 16SrRNA (Table 3 and Figure 1). The involvement of *Lactobacillus* spp. in the production of Nigerian indigenous fermented foods including those isolated in the present study have been widely reported (Obafemi et al., 2022). *Lactobacillus fermentum* was among the LAB isolated from “ogi”, “fufu”, “garri” and “nunu” by Ikwemesi et al. (2017). Reports on the use of *Lactobacillus parafarraginis* and *L. buchneri* generally in food fermentation, and particularly in Nigerian indigenous fermented foods is scarce. *Lactobacillus parafarraginis* is used as an additive in silages due to the preservative and probiotic properties (Xu et al., 2017; Bampidis et al., 2020) and Cheon et al. (2020) isolated *L. buchneri* from Korean fermented foods.

Among the other bacteria isolated in this study, five, designated as B1, B2, B3, B4 and B5 were selected due to their susceptibility to the CFE of the LAB isolates, characterized and identified as *Bacillus anthracis* CHF-2011-32b, *Providencia vermicola* KSVDL-09-71600, *Bacillus cereus* DHM5-1, *Providencia rettgeri* SIRT3 and *Providencia stuartii* SILT11 (Table 3). *Bacillus* spp. produce amylase and proteases making them important fermenting organisms in the production of many indigenous fermented foods particularly in condiments such as fermented locust beans “iru” (Saliu et al., 2019), oil bean seed “ugba” (Olasupo et al., 2016) and seeds of *Prosopis africana* “okpehe” (Oguntoyinbo et al., 2010). *Providencia* species are commonly found in water, soil and animals and may have contaminated the food samples from any of these sources. They are opportunistic pathogens of the urinary tract infection (UTI), and are the most common cause of catheter-associated UTIs especially in the elderly (Armbuster et al., 2014)

Antimicrobial Activity of the Cell-free extracts of the Lactic Acid Bacterial Isolates

The CFE of the isolated LAB, their derivatives after neutralizing, and after precipitating using ammonium sulphate $\{(NH_4)_2SO_4\}$, inhibited the test organisms with inhibition zones diameters ranging from 5.25 ± 0.34 mm to 25.33 ± 2.52 mm (Table 5). All the test organisms except *Klebsiella oxytoca*, were inhibited by the CFE

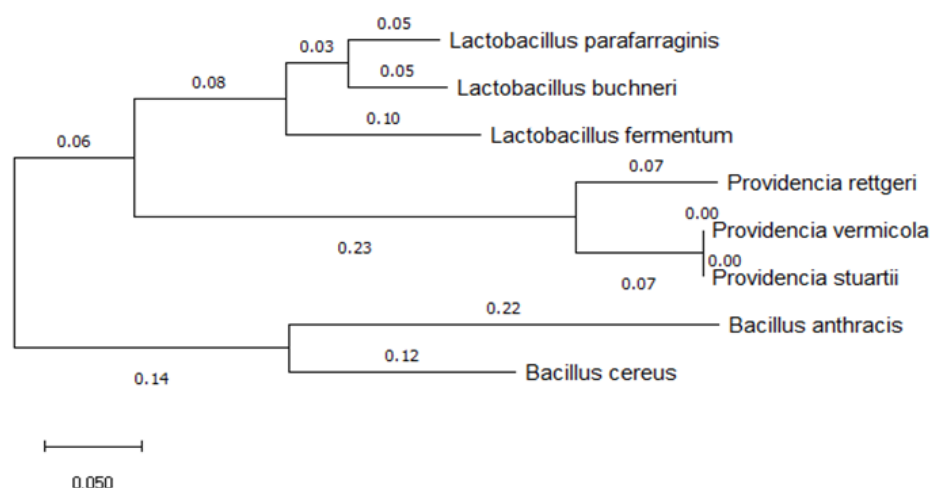


Figure 1: Neighbor-joining tree analysis of the evolutionary distance and phylogenetic relationship of Bacteria Isolated from Nunu.

Table 2: Colony morphology, appearance under the microscope after staining, and biochemical characteristics of bacteria isolates

Isolates	Colony Morphology						Cell Morphology						Biochemical Characteristics																	
	Shape	Size	Pigmentation	Margin	Elevation	Colony surface	Optical character	Consistency	Gram stain	Cell shape	Arrangements	Spore	Catalase	Oxidase	Citrate	Urease	Indole	Methyl Red	Voges Proskauer	Starch	H ₂ S	Gas	Glucose fermentation	Motility	Lactose	Sucrose	Fructose	Maltose	Growth at 45°C	Growth in 6.5% NaCl
L2	I	S	W	L	F	Sm	O	Bt	+	Rd	Sn	-	-	-	-	-	+	+	-	-	+	-	AK	-	AG	-	A	AG	+	-
L11	C	S	Cr	E	F	Sm	O	Bt	+	Rd	P	-	-	-	-	-	-	-	-	-	-	-	AA	-	A	A	A	A	-	-
L13	C	S	Cr	E	R	Sm	O	Bt	+	Rd	Ch	-	-	-	-	-	-	-	-	+	-	-	KA	-	AG	AG	AG	A	+	-
B1	C	M	W	L	F	Rg	T	Bt	+	Rd	Ch	+	+	-	+	-	-	-	+	+	+	+	AA	+	-	A	AG	AG	+	+
B2	C	S	W	L	R	Rg	O	Bt	-	Rd	Sn	-	+	-	+	+	+	+	-	-	-	-	KA	+	A	A	A	A	+	+
B3	C	S	W	E	F	Rg	T	Bt	+	Rd	P	+	+	-	+	-	+	+	+	+	-	-	AA	-	-	A	A	A	+	+
B4	I	S	Cr	E	F	Rg	Tp	Bt	-	Rd	Sn	-	+	-	-	+	+	+	-	-	-	+	AA	+	A	A	AG	AG	+	+
B5	C	S	Cr	E	R	S	T	Bt	-	Rd	Cl	-	+	-	-	+	+	+	-	+	-	-	AA	+	A	A	A	A	+	+

Shapes were Circular (C) and Irregular (I), colony sizes that were less than 5 mm were taken as small (S) while sizes above 5 mm were taken as medium (M), the colors of the colonies were White (W) and Cream (Cr), margins were Entire (E) and Lobate (L). For elevation, F = flat and R = Raised, Smooth (Sm) and Rough (Rg) colony surfaces occurred and the colonies were translucent (Tp), transparent (T) and opaque (O). All the isolates were butyrous (Bt). “+” sign denotes that the test gave positive result while negative result is indicated with “-“ sign. The cell shape was rod (Rd) for all, cell arrangements include: Chains (Ch), Clusters (Cl), Pairs (P) and Single (Sn). For sugar fermentation, AK = Acid/Alkaline, KA = Alkaline/Acid, AA = Acid/Acid, A: Acid, AG: Acid and gas.

Table 3: Blastn summary of 16SrRNA sequenced data of bacteria isolate showing the names of the organisms with percentage identity and accession numbers

Isolate	Organism	Max Score	Identity (%)	Accession Number
L2	<i>Lactobacillus parafarraginis</i> OCLP1	1485	93.99	MK 605951.1
L11	<i>Lactobacillus fermentum</i> CSL58	1411	94.58	KT 800406.1
L13	<i>Lactobacillus buchneri</i> MZE 12	1445	94.89	KR 055498.1
B1	<i>Bacillus anthracis</i> CHF-2011-32b	470	85.19	KC 136582.1
B2	<i>Providencia vermicola</i> KSVDL-09-71600	1317	94.08	HM 071065.1
B3	<i>Bacillus cereus</i> DHM5-1	767	86.00	KF 625179.1
B4	<i>Providencia rettgeri</i> SIRT3	1306	93.62	MK 780046.1
B5	<i>Providencia stuartii</i> SILT11	1123	89.50	MK 780040.1

of *L. parafarraginis* OCLP1. Its activities against the clinical test organisms as well as those isolated from food samples indicate that extracts from the organism can serve both preservative and therapeutic roles. In contrast, the CFE of *L. fermentum* CSL58 inhibited only two of the clinical isolates, *K. oxytoca* and *Salmonella enterica* along with four of the food isolates and can therefore be associated more with preservative rather than therapeutic role. The CFE of *L. buchneri* MZE 12 inhibited five of the clinical isolates and three of the organisms isolated from food items. In all, the inhibition zones were lower than those obtained from the CFE of *L. parafarraginis* OCLP1. This indicates diverse role close to that of *L. parafarraginis* OCLP1 but with lower inhibitory properties. *Lactobacillus* species are known to produce antimicrobial activities against related and unrelated organisms. *L. parafarraginis* stands out as inhibitors of multidrug resistant bacteria including *Providencia* spp. (Armbuster et al., 2014). Inhibition of multidrug resistant pathogenic bacteria, e.g., carbapenem resistant members of the Enterobacteriaceae (Chen et al., 2019) occur in many other *Lactobacillus* spp.

In general, LAB produce lactic acid as a major antimicrobial compound responsible for their inhibitory activities. Additionally, some produce other compounds including bacteriocins

which contribute immensely to their antimicrobial functions (Tang et al., 2023) The LAB isolates in this study have shown evidence of the involvement of compounds other than lactic acid as the antimicrobial factor (Table 4). The inhibitory activity of the ammonium sulphate precipitated protein from the CFE of the LAB is a proof that bacteriocin was produced. Bacteriocin production by *L. fermentum* (Henedia-Castro et al., 2021), *L. parafarraginis* (Allen-McFarlane et al., 2018) and *L. buchneri* (Mahdi et al., 2017) were previously reported.

Spectrum of Antimicrobial Activities of the Lactic Acid Bacterial Isolates

All the LAB in this study demonstrated inhibitory activities against both Gram-positive and Gram-negative bacteria used to determine the spectra of activities. Among the LAB, *L. parafarraginis* OCLP1 inhibited 14 out of 17 organisms including the yeast, *Candida albicans* while the other two inhibited 8 each (Table 5). Similarly, Djadouni & Kihalil (2012) reported a *Lactobacillus* sp. able to inhibit ten test bacteria that are both Gram-positive and Gram-negative. Also, da Silva et al. (2023) obtained a broad-spectrum antimicrobial from *L. acidophilus*.

Table 4: Mean diameter zone of inhibition (mm $\pm$ standard deviation) of test organisms by crude, Sodium hydroxide neutralized and Ammonium sulphate precipitants of the cell-free extracts of *Lactobacillus parafarraginis* OCLP1; *L. fermentum* CSL58; L13: *L. buchneri* MZE 12

Test Organisms	Zone of Inhibition (mm)								
	CCFS			NCFS			PCFS		
	L2	L11	L13	L2	L11	L13	L2	L11	L13
B1	12.00 $\pm$ 1.73 ^a	11.33 $\pm$ 1.15 ^b	11.00 $\pm$ 1.00 ^a	8.33 $\pm$ 0.58 ^b	6.33 $\pm$ 0.54 ^b	10.00 $\pm$ 1.00 ^c	12.00 $\pm$ 1.00 ^b	11.33 $\pm$ 0.58 ^a	7.00 $\pm$ 1.00 ^a
B2	10.67 $\pm$ 0.58 ^a	7.67 $\pm$ 0.58 ^a	0.00	7.17 $\pm$ 0.76 ^b	9.00 $\pm$ 0.00 ^c	0.00	18.33 $\pm$ 0.58 ^d	11.67 $\pm$ 0.58 ^a	0.00
B3	13.33 $\pm$ 4.51 ^b	10.33 $\pm$ 0.58 ^b	13.00 $\pm$ 0.00 ^a	10.33 $\pm$ 0.58 ^c	7.00 $\pm$ 0.00 ^b	8.00 $\pm$ 1.00 ^b	17.67 $\pm$ 0.58 ^c	14.33 $\pm$ 0.58 ^b	8.33 $\pm$ 0.58 ^a
B4	17.00 $\pm$ 2.00 ^c	0.00	0.00	13.00 $\pm$ 1.00 ^d	0.00	0.00	10.33 $\pm$ 0.58 ^b	0.00	0.00
B5	12.00 $\pm$ 0.00 ^a	13.00 $\pm$ 0.00 ^c	12.00 $\pm$ 1.00 ^a	6.33 $\pm$ 0.58 ^a	5.00 $\pm$ 1.00 ^a	8.33 $\pm$ 0.57 ^b	25.33 $\pm$ 2.52 ^c	24.67 $\pm$ 1.53 ^c	7.33 $\pm$ 0.57 ^a
YE	20.33 $\pm$ 5.69 ^d	0.00	18.33 $\pm$ 1.53 ^b	13.33 $\pm$ 1.09 ^d	0.00	7.32 $\pm$ 0.62 ^a	18.33 $\pm$ 1.69 ^d	0.00	18.33 $\pm$ 1.53 ^c
SE	20.33 $\pm$ 5.69 ^d	18.00 $\pm$ 2.00 ^d	18.33 $\pm$ 1.53 ^b	5.67 $\pm$ 1.15 ^a	0.00	10.24 $\pm$ 0.25 ^c	15.67 $\pm$ 2.15 ^c	15.15 $\pm$ 0.91 ^b	14.25 $\pm$ 1.33 ^b
CA	13.00 $\pm$ 5.00 ^b	0.00	0.00	7.03 $\pm$ 0.33 ^b	0.00	0.00	9.00 $\pm$ 0.65 ^b	0.00	0.00
SL	14.67 $\pm$ 4.51 ^b	0.00	0.00	10.25 $\pm$ 0.57 ^c	0.00	0.00	7.25 $\pm$ 0.55 ^a	0.00	0.00
CF	20.33 $\pm$ 3.51 ^d	0.00	18.67 $\pm$ 1.16 ^b	7.63 $\pm$ 1.22 ^b	0.00	0.00	16.66 $\pm$ 1.56 ^c	0.00	13.40 $\pm$ 1.28 ^b
SA	16.33 $\pm$ 4.93 ^c	0.00	0.00	5.25 $\pm$ 0.34 ^a	0.00	0.00	12.81 $\pm$ 1.54 ^b	0.00	0.00

Values are means of triplicate determination $\pm$ SD. Means on the same column with different superscripts are significantly different ($p < 0.05$). CCFS = Crude Cell-free supernatant; NCFS Sodium hydroxide neutralized CCFS; PCFS = Ammonium sulphate precipitant of CCFS L2: *Lactobacillus parafarraginis* OCLP1; L11: *L. fermentum* CSL58; L13: *L. buchneri* MZE 12; B1 – B5, *Bacillus anthracis* CHF-2011-32b, *Providencia vermicola* KSVDL-09-71600, *Bacillus cereus* DHM5-1, *Providencia rettgeri* SIRT3 and *Providencia stuartii* SILT11 respectively; YE; *Yersinia enterocolitica*, SE; *Salmonella enterica*, CA; *Candida albicans*, SL; *Serratia liquifaciens*, CF; *Citrobacter freundii*, SA; *Staphylococcus aureus*.

Plasmid Mediated Antimicrobial Activity of the Lactic Acid Bacterial Isolates

After treatment sodium dodecyl sulphate for plasmid curing, *L. parafarraginis* OCLP1, did not inhibit *P. stuartii* SILT11 that was used as the test organism due to previous susceptibility. This indicates that the bacteriocin may be plasmid mediated in agreement with Dodd et al., (1990) and could be that the gene/s have been previously transferred between the chromosome and plasmid since according to Yang et al. (2014), bacteriocins of Gram-positive bacteria are chromosomal. On the other hand, the bacteriocins of *L. fermentum* CSL58 and *L. buchneri* MZE 12 are chromosomal because the test organisms were inhibited after plasmid curing (Table 6).

CONCLUSIONS

This study concluded that lactic acid bacteria that have antibacterial activities and are capable of producing bacteriocins can be isolated from “nunu”. The study therefore recommends further improvement of the bacteriocin-producing lactic acid bacterial isolates for use

as starter cultures in milk fermentation after ensuring its safety through toxicological studies.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 5: Average diameter of inhibition zones (mm $\pm$ standard deviation) observed for test organisms challenged with cell-free extracts of *Lactobacillus parafarraginis* OCLP1; *L. fermentum* CSL58 and *L. buchneri* MZE 12.

Test Organisms	Diameter of Zones of Inhibition (mm)		
	<i>Lactobacillus parafarraginis</i>	<i>L. fermentum</i>	<i>L. buchneri</i>
<i>Bacillus anthracis</i> CHF-2011-32b	12.00 $\pm$ 1.73	11.33 $\pm$ 1.15	11.00 $\pm$ 1.00
<i>Providencia vermicola</i> KSVDL-09-71600	10.67 $\pm$ 0.58	7.67 $\pm$ 0.58	0.00
<i>Bacillus cereus</i> DHM5-1	13.33 $\pm$ 4.51	10.33 $\pm$ 0.58	13.00 $\pm$ 0.00
<i>Providencia rettgeri</i> SIRT3	17.00 $\pm$ 2.00	0.00	0.00
<i>Providencia stuartii</i> SILT11	12.00 $\pm$ 0.00	13.00 $\pm$ 0.00	12.00 $\pm$ 1.00
<i>Yersinia enterocolitica</i>	20.33 $\pm$ 5.69	0.00	18.33 $\pm$ 1.53
<i>Salmonella enteritis</i>	20.33 $\pm$ 5.69	18.00 $\pm$ 2.00	18.33 $\pm$ 1.53
<i>Klebsiella oxytoca</i>	0.00	24.00 $\pm$ 5.29	0.00
<i>Candida albicans</i>	13.00 $\pm$ 5.00	0.00	0.00
<i>Escherichia coli</i>	20.00 $\pm$ 2.00	0.00	18.00 $\pm$ 2.00
<i>Serratia liquifaciens</i>	14.67 $\pm$ 4.51	0.00	0.00
<i>Bacillus subtilis</i>	9.33 $\pm$ 6.11	0.00	16.67 $\pm$ 4.16
<i>Citrobacter freundii</i>	20.33 $\pm$ 3.51	0.00	18.67 $\pm$ 1.16
<i>Staphylococcus aureus</i>	16.33 $\pm$ 4.93	0.00	0.00
<i>Vibrio cholerae</i>	0.00	6.00 $\pm$ 1.02	7.00 $\pm$ 1.00
<i>Pseudomonas aeruginosa</i>	0.00	9.00 $\pm$ 1.52	0.00
<i>Bacillus megaterium</i>	12.00 $\pm$ 2.00	0	0

Values are means of triplicate determination $\pm$ SD.

Table 6: Inhibition of *Providencia stuartii* SILT11 by cell-free extracts of Sodium Dodecyl Sulphate Treated- *Lactobacillus parafarraginis* OCLP1, *L. fermentum* CSL58 and *L. buchneri* MZE 12 for the Detection of Plasmid Mediated Bacteriocins.

LAB Isolates	Test Organisms	Inhibition Profile
<i>Lactobacillus parafarraginis</i> OCLP1	<i>Providencia stuartii</i> SILT11	-
<i>Lactobacillus fermentum</i> CSL58	<i>Providencia stuartii</i> SILT11	+
<i>Lactobacillus buchneri</i> MZE 12	<i>Providencia stuartii</i> SILT11	+

The sign “+” denotes inhibition of the test organism while “-” denotes organism was not inhibited.

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