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-RESEARCH ARTICLE-

Partial Purification, Characterization and Application of Bacteriocin from Bacteria Isolated *Parkia biglobosa* Seeds

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Abstract

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains. Fermented *Parkia biglobosa* seeds (African locust bean) were screened for bacteriocin-producing lactic acid bacteria (LAB) with the characterization of putative bacteriocins. Bacteriocin-producing lactic acid bacteria (LAB) were identified by 16s rDNA sequencing. Molecular sizes of the bacteriocins were determined using the tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (tricine-SDS–PAGE) and effects of enzymes, pH, detergents and temperature on bacteriocin activity investigated, using standard procedures. Bacteriocins production and activities were measured by spectrophotometric analysis. Statistical analysis was carried out using student t-test and Analyses of Variance. Bacteriocigenic LAB isolated were *Lactobacillus plantarum* Z1116, *Enterococcus faecium* AU02 and *Leuconostoc lactis* PKT0003. They inhibited the growth of both Gram-positive and Gram-negative bacteria. The sizes of bacteriocins Z1116, AU02 and PKT0003 were 3.2 kDa, 10 kDa and 10 kDa, respectively. The synergistic effects of characterized bacteriocins and rifampicin tested on organisms showed significant differences ($P < 0.05$), as compared with the effects of only one of the two. The antimicrobial activity of the three bacteriocins was deactivated after treatment of the cell-free supernatants with proteinase K, papain, pepsin and trypsin. *Parkia biglobosa* seeds are, therefore, rich in LAB bacteriocins which could be explored. The biosynthetic mechanisms of LAB bacteriocins could be employed in food safety and security, preservation, peptide design, infection control and pharmacotherapy. This should help in the control of undesirable bacteria and in designing more potent and selective antimicrobial peptides.

Keywords:

African locust bean, antimicrobial peptides, bacteria, bacteriocins, *Parkia biglobosa*

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Introduction

Fermented *Parkia biglobosa* seeds (*Irú*) is a type of fermented locust beans used as a condiment in cooking. It is similar to ogiri and douchi. It is very popular among the Yoruba people of Nigeria. It can be found fresh or dried. The fresh variety is usually wrapped in moimoi leaves, which are similar in appearance and texture to banana leaves. It has a pungent smell. The dried variety is flattened into discs or cakes for sale. Dried fermented *Parkia biglobosa* seeds (*Irú*) is weaker in flavor and pungency than fresh (though frying it in cooking oil will restore much of the flavor). The dried variety stores very well in freezers (Ogunshe *et al*., 2008).

According to Campbell-Platt (1980), *Iru* or dawadawa (the most popular food condiment in the entire of savannah region of the West and Central Africa) are respective Yoruba and Hausa names for fermented seed cotyledons of African locust beans (*Parkia biglobosa*). It is an indigenous tree species that is economically and socially important for local people in sub-Saharan Africa (Teklehaimanot, 2004).

The fermentation of *Parkia biglobosa* seeds by *Bacillus* species is an example of an alkaline fermentation process (Achi, 2005), and after fermentation for 72-96 hours, the cotyledons become soft and dark with a strong characteristic ammoniacal odour (Odunfa, 1986; Achi, 1992). Furthermore, over-fermentation has been known to be capable of generating unacceptable levels of volatile fatty acids (Achi, 2005), while improper storage has also been known to cause maggot infestation and microbial contamination including antibiotic resistant bacteria.

With the passage of time, non judicious use of antibiotics became common practice resulting in the reemerging infections. For solution, the derivatives of antibiotics were discovered and applied but pathogenic bacteria developed the resistance against them also. Now it was the need of time to search for new strategies for infection control of resistant pathogenic bacteria. Nowadays, consumers are aware of the health concerns regarding food additives; the health benefits of "natural" and "traditional" foods, processed without any addition of chemical preservatives, are becoming more attractive.

Refocusing attention on food security is essential in the 21st century with a universal call to feed the hungry, and especially on the impact of biopatenting on poor communities who are the primary victims of hunger in our world. Antibacterial metabolites of lactic acid bacteria, such as bateriocins, have potential as natural preservatives to control the growth of spoilage and pathogenic bacteria in food. Bacteriocin is useful as a preservative in food due to its heat stability, wider pH tolerance and its proteolytic activity. Bacteriocins are ribosomally synthesized peptides originally defined as proteinaceous compound affecting growth or viability of closely related organisms (Gautam & Sharma, 2009; Bello *et al*., 2016a).

The primary metabolite nature of bacteriocins is their most significant advantage over conventional antibiotics since they have relatively simple biosynthetic mechanisms compared with conventional antibiotics, which are secondary metabolites. This makes bacteriocins easily amenable through bioengineering to increase either their activity or specificity towards target microorganisms (Perez *et al*., 2014). The aim of this study was to partially purify and characterize bacteriocins from bacteria isolated from *Parkia biglobosa* seeds with the evaluation of their applications as antimicrobial agents.

Materials and Methods

Sources of Samples

One hundred samples of fermented *Parkia biglobosa* seeds were purchased from local sellers in different markets which were Awolowo, Sagamu; Sabo, Sagamu; Falawo, Sagamu; Kuto, Abeokuta; Ago-Iwoye; Atikori, Ijebu-Igbo; Mamu, Ogun State; New market, Ijebu-Ode; Oke-Aje; Ijebu-Ode; Tokin, Lagos; Sabo, Ikorodu; Igbogbo, Ikorodu; Ketu, Lagos; Oshodi, Lagos; Bariga, Lagos and Shomolu, Lagos. New Gbagi, Ibadan; Orita-challenge, Ibadan; Bodija, Ibadan; Agbeni-Ogunpa, Ibadan; Ojoo, Ibadan; Olodo, Ibadan; Aleshinloye, Ibadan; Iyana-Church, Ibadan; Iwo, Iwo.

Assaying for bacteriocin-producing lactic acid bacteria

Samples of *Parkia biglobosa* seeds were blended with electric blender (HR 28151, Netherland) and serial dilutions made and plated onto MRS agar (Biolab, Biolab Diagnostics, South Africa) supplemented with 50 mg/l Delvocid (Gist-brocades, B.V., Delft, The Netherlands). Colonies were covered with a second layer of MRS agar containing the same concentration of Delvocid. The plates were incubated anaerobically (OXOID, Gas Generation Kit, Hampshire, England) at 30^{O}C for 48 h. Plates with 50 or less colonies were covered with BHI medium containing 1.0% (m/v) agar (Merck, Darmstadt, Germany) and inoculated with *Enterococcus faecium* HKLHS (final concentration level of 10^6 CFU ml^{-1}). The plates were incubated for 24 h at 30^{O}C. Colonies with inhibition zones were selected, cultured in MRS broth (Biolab) and tested for antimicrobial activity against *E. faecium* HKLHS and *Lactobacillus sakei* DSM 20017 by using the agar-spot test and disc diffusion methods (Todorov & Dicks, 2005a). The antimicrobial effect of lactic acid was eliminated by adjusting the pH of the supernatants to 6.0 with sterile 1 M NaOH. Activity was expressed as arbitrary units (AU) ml^{-1}. One AU was defined as the reciprocal of the highest serial two fold dilution showing a clear zone of growth inhibition of the indicator strain (Bello *et al*., 2016a,b).

Identification of isolates with antimicrobial activities

Isolates with antimicrobial activity against *L. sakei* DSM 20017 and *E. faecium* HKLHS were selected and identified to genus-level according to their physiological and biochemical characteristics as described in a report by Bello *et al*. (2016a). Species-specific PCR included primers for *Leuconostoc lactis* (Llac F: 5′-AGG CGG CTT ACT GGA CAA C-3′ and Llac-R: 5′-CTT AGA CGG CTC CTT CCA T-3′; Lee *et al.*, 2000), *Lactobacillus plantarum* (plan F: 5′-CCG TTT ATG CGG AAC ACC TA-3′ and REV: 5′-TCG GGA TTA CCA AAC ATC AC-3′; Torriani *et al.*, 2001), and genus-specific primers for *Enterococcus* spp (Ent1: 5′-TAC TGA CAA ACC ATT CAT GAT G-3′ and 5′-AAC TTC GTC ACC AAC GCG GAA C-3′; Ke *et al*., 1999). The universal primers 8f (5′-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3′) and 1512r (5′-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT), where Y indicates C + T and M indicates A + C, were used to amplify the 16S rDNA gene according to Felske *et al.* (1997).

Amplified fragments with the correct sizes were cloned into pGEM$^{\infty}$-T Easy Vector (pGEM∞-T Easy Vector Systems, Promega, Madison, USA) and transformed to *E. coli* DH5α.

Transformed cell suspensions (100 µl) were plated onto Luria Bertani agar (Biolab), supplemented with ampicillin (100 µgml^{-1}), X-gal and IPTG. After 12 h of incubation at 37^{0}C, transformants were selected and plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen∞, Valencia, California, USA). DNA was sequenced using the bigdye™ terminator cycle chemistry (Biosystems, Warrington, England) on an ABI Genetic Analyzer 3130XL Sequencer (Applied Biosystem, SA, Pty, Ltd.) (Felske *et al*., 1997; Bello *et al*., 2016b).

Bacteriocin production

MRS broth (Biolab) was inoculated with a 24-h-old culture (2%, v/v) of a bacteriocin-producing strain (each of *Lactobacillus plantarum* Z1116, *Enterococcus faecium* AU02 and *Leuconostoc lactis* PKT0003). Incubation was at 30^{O}C. Antimicrobial activity (AU ml^{-1}) of the bacteriocins, and changes in pH and optical density (at OD_{600} nm) of the cultures, were determined at 3 h and 1 h intervals for 24 h. *E. faecium* HKLHS (10^{6} CFU ml^{-1}) was used as sensitive strain. In addition, forty-five bacterial strains were used in the determination of spectra of activity. They were cultured in MRS or BHI (Biolab) broth at 30^{O}C or 37^{O}C, respectively (Torriani *et al.*, 2001).

Molecular size of the bacteriocins

Young actively growing cultures were centrifuged for 15 min at 10,000g and the pH was corrected to 6.0 with 6 M NaOH. To prevent proteolytic degradation of the bacteriocins, cell-free supernatants were treated for 10 min at 80^{O}C. Ammonium sulfate was added slowly to the cell-free supernatants to 60% saturation for *Lactobacillus plantarum* Z1116, *Enterococcus faecium* AU02 and *Leuconostoc lactis* PKT0003, stirred for 4 h at 4^{O}C and then centrifuged (10,000g, 1 h, 4^{O}C). The precipitates were re-suspended in 10 ml 25 mM ammonium acetate buffer (pH 6.5) and desalted by dialysis (1000 Da cut-off dialysis membrane, Spectrum Inc., CA, USA). Further separation was by tricine–SDS–PAGE, as described by Schägger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 2.5 to 45.0 kDa (Amersham Biosciences Europe GmbH, Freiberg, Germany) was used. The gels were fixed and one half stained with Coomassie Blue R250 (Saarchem, Krugersdorp, South Africa). The position of the active bacteriocin was determined in the unstained gel, as described by Bello *et al.* (2016b). *E. faecium* HKLHS or *L. sakei* DSM 20017 (10^{6} CFU ml^{-1}), suspended in MRS broth (Biolab) supplemented with 1% (m/v) agar, was used as a sensitive strain.

Effects of enzymes, pH, detergents and temperature on bacteriocin activity

Cell-free supernatants of bacteriocin-producing strains, obtained by centrifugation (8000g, 10 min, 4^{O}C), were adjusted to pH 6.0 with 1 M NaOH. Two mils of samples were incubated for 2 h in the presence of 1.0 or 0.1 mg ml^{-1} (final concentration) trypsin (Roche, USA), pronase (Roche), Proteinase K (Roche), pepsin (Roche), papain (Roche) and α-amylase (Roche) and then tested for antimicrobial activity using the agar-spot test method. In a separate experiment, the effect of sodium dodecyl sulphate (SDS), Tween 20, Tween 80, urea, Triton X-100, Triton X-114 and Na–EDTA on bacteriocins in cell-free supernatants was determined as described by Todorov and Dicks (2006a). The effect of pH on the bacteriocins was determined by adjusting the cell-free supernatant from pH 2.0 to 12.0 at intervals of half, with sterile 1 M HCl or 1 M NaOH. After 2 h of incubation at 30^{O}C, the samples were readjusted to pH 6.5 with sterile 1 M HCl or 1 M NaOH and the activity was determined as described before (Bello et al., 2016a, 2016b). The effect of temperature on the bacteriocins was tested by heating the cell-free supernatants to 30^{O}, 37^{O}, 45^{O},

60^{O} and 100^{O}C, respectively. Residual bacteriocin activity was tested after 30, 60 and 120 min at each of these temperatures, as described before (Todorov *et al.*, 2006).

Response of isolates to bacteriocins

A 10 ml aliquot of bacteriocin-containing filter-sterilized (0.20 μm, Minisart∞, Sartorius, USA) supernatant (pH 6.0) was added to a 100 ml culture of *L. sakei* DSM 20017 or *E. faecium* HKLHS in an early exponential phase (OD_{600} = 0.12) and incubated for 15 h. Optical density readings (at 600 nm) were recorded at 1-h intervals. In a separate experiment, extracellular levels of β-galactosidase activity were monitored. Eleven-h-old cultures of *E. faecium* HKLHS and *L. sakei* DSM 20017 (80 ml each) were harvested and the cells were washed twice with 0.03 M sodium phosphate buffer (pH 6.5) and re-suspended in 16 ml of the same buffer. Two millilitres of each cell suspension were treated with 2 ml of bacteriocins Z1116, AU02 and PKT0003 respectively, for 5 min at 25^{0}C, followed by the addition of 0.2 ml 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranoside, Fluka, USA) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 37^{O}C, the reaction of β-galactosidase was stopped by the addition of 2.0 ml 0.1 M sodium carbonate. The cells were harvested (8000g, 15 min, 25^{0}C) and the absorbance readings of the supernatant were recorded at 420 nm. Cells disrupted with 0.1 mm diameter glass beads vortexed (for 5 min) served as control (Todorov *et al.*, 2006).

Adsorption study of the bacteriocin to the producer cells

This was studied according to the method described by Yang *et al*. (1992). After 18 h of growth at 30^{O}C, the culture was adjusted to pH 6.0, the cells were harvested (10,000g, 15 min, 4^{O}C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The cells were re-suspended in 10 ml 100 mM NaCl (pH 2.0), stirred for 1 h at 4^{O}C and then harvested (12,000g, 15 min, 4^{O}C). The cell-free supernatant was neutralized to pH 7.0 with sterile 1 M NaOH and tested for activity as previously described. The percentage adsorption of bacteriocins to the target cells was calculated according to the following formula:

$$\% \text{ adsorption} = 100 - \left(\frac{\text{bacteriocin activity after treatment}}{\text{original bacteriocin activity}} \times 100\right)$$ (Vaucher *et al.*, 2011)

Statistical analysis of data obtained

Paired-Samples T-test and One-way Analysis of Variance (ANOVA) were used to determine the statistical difference between the microbial activity of bacteriocins and antibiotic (rifampicin) against *L. monocytogens* NCTC 4885 using SPSS version 17.0.

Results

Table 1 shows the lactic acid bacterial genera isolated from *Parkia biglobosa*. Bacteriocigenic LAB strains were *Lactobacillus plantarum* Z1116, *Enterococcus faecium* AU02 and *Leuconostoc lactis* PKT0003 while other LAB isolates were *Lactobacillus plantarum, L. fermentum* and *L. pentosus.*

Table 1. Bacterial genera isolated from protein-rich foods from *Parkia biglobosa*

Bacteriocigenic LAB	Other LABs
Lactobacillus plantarum Z1116,	*Lactobacillus plantarum,*
Enterococcus faecium AU02	*L. fermentum*
Leuconostoc lactis PKT0003	*L. pentosus*

Table 2 shows the spectra of activities of *L. plantarum* Z1116, *E. faecium* AU02 and *L. lactis* PKT0003 against certain Gram-positive and Gram-negative bacterial strains. Out of 45 indicator bacterial strains investigated for their sensitivity to the different bacteriocin-like inhibitory substances, 27 (60%) were sensitive (positive) to Z1116, 25 (55.56%) were positive to AU02 and 15 (33.33%) sensitive to PKT0003.

Table 2. Antibacterial spectrum of activity of bacteriocins produced by *L. plantarum* Z1116, *E. faecium* AU02 and *L. lactis* PKT0003 against bacterial indicator strains

Bacterial Strain	Medium	Temperature (^{O}C)	Bacteriocin-like inhibitory activity		
			Z1116	AU02	PKT0003
Enterococcus faecalis 1071	MRS[g]	30	+	-	-
					-
E. faecalis E88	MRS	30	-	+	+
E. faecalis E90	MRS	30	-	+	-
E. faecalis E92	MRS	30	-	+	-
E. faecalis ET05[a]	MRS	30	+	+	-
E. faecalis ET12[a]	MRS	30	+	-	-
E. faecalis ET88[a]	MRS	30	+	-	+
E.faecium HKLHS	MRS	30	+	+	-
E. faecium T8	MRS	37	+	+	-
E. faecalis PTA-7278 (ST4SA)[b]	MRS	30	+	+	+
					-
Escherichia coli P40	BHI[h]	37	+	+	+
					+
E. coli P46	BHI	37	-	-	-
E. coli P8	BHI	37	-	-	-
Klebsiella pneumoniae P30	BHI	37	+	+	-
					-

Lactobacillus casei defensis	MRS	30	-	-	-
L. casei Shirota	MRS	30	-	-	-
L. curvatus DF38	MRS	30	+	-	-
L. curvatus ET34[a]	MRS	30	-	-	-
L. curvatus ET06[a]	MRS	30	-	-	-
L.delbruekii ET32[a]	MRS	30	+	-	+
L. jonhsonii Lc1	MRS	30	+	+	-
L.jonhsonii VPI1830	MRS	30	+	-	
L.plantarum ST202Ch	MRS	30	+	-	
L. rhamnosus Lgg	MRS	30	+	+	
L. sakei DSM 20017[c]	MRS	30	-	+	
L. salivarius 241 MRS	MRS	30	+	-	
Lactococcus lactis subsp. *lactis* HV219	MRS	37	-	-	-
L. innocua LMG 13568[d]	BHI	37	+	+	+
L. monocytogenes NCTC 4885[e]	BHI	37	+	+	+
L. monocytogenes ScottA	BHI	37	+	+	-
L. monocytogenes NCTC 11944[e]	BHI	37	+	+	-
P. aeruginosa P22	BHI	37	-	-	+
P. aeruginosa P7	BHI	37	+	+	-
Pseudomonas spp P28	BHI	37	+	+	+
S. aureus P13	BHI	37	+	+	-
S. aureus P36	BHI	37	+	-	+
S. aureus P37	BHI	37	-	-	-
S. aureus P38	BHI	37	-	+	+
Staphylococcus uberis P12[f]	BHI	37	+	-	+
Streptococcus agalactiae P9	BHI	37	-	-	-

Bacterial Strain	Medium	Temperature (^{O}C)	Bacteriocin-like inhibitory activity		
			Z1116	AU02	PKT0003

Streptococcus caprinus ATCC 700065[a]	BHI	37	-	+	-
S. caprinus ATCC 700066[b]	BHI	37	-	+	-
Streptococcus faecalis P20	BHI	37	+	+	+
Streptococcus spp TL2R	BHI	30	-	+	-

Keys: – = No activity; + = inhibition zone.

[a] Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal.

[b] ATCC: American Type Culture Collection, Manassas, VA, USA.

[c] DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

[d] LMG: Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.

[e] NCTC: National Collection of Food Bacteria, Reading, UK.

[f] UWC: Department of Microbiology, University of Western Cape, Cape Town, South Africa.

[g] De Man, Rogosa and Sharpe.

[h] Brain Heart Infusion.

All other strains were from Department of Biological Sciences, Faculty of Science and Technology, North-West University, Mafikeng, South Africa.

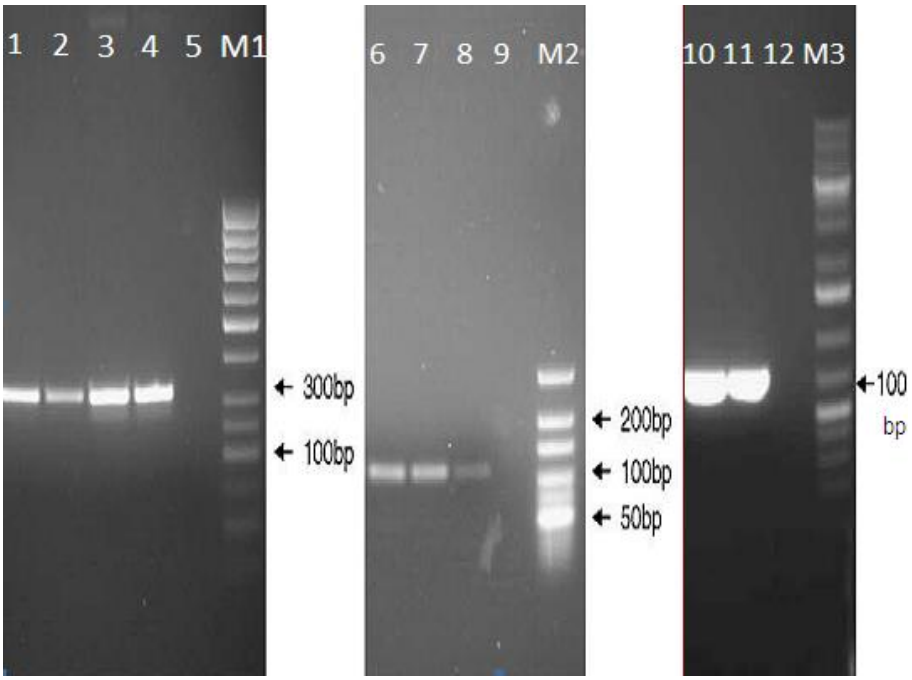

Figure 1: Agarose gels showing DNA fragments obtained after PCR with species-specific and genus-specific primers. Lanes 1 and 2: strain Z1116, lanes 3 and 4: *L. plantarum* NCBF 363, lane 5: no DNA loaded, lane M1: O'GeneRuler™ 100-bp DNA Ladder from Fermentas, lanes 6 and 7: strain AU02, lane 8: *E. faecalis* PTA-7278 (ST4SA), lane 9: no DNA loaded, lane M2:

O'GeneRuler™ Ultra Low Range DNA Ladder from Fermentas, lane 10: strain PKT0003, lane 11: *L. lactis* NCDO 533 (DSM 20202T), lane 12: no DNA loaded and lane M3: O'GeneRuler™ 1 kb DNA Ladder (Fermentas).

Figure 1 shows agarose gels of DNA fragments obtained after PCR with species-specific and genus-specific primers. Strain Z1116 did not produce gas from glucose but fermented carbohydrates and this is typical of *L. plantarum*. DNA amplification yielded a fragment identical in size to that reported for *L. plantarum*. Strain AU02 does not produce gas from glucose, ferments the same sugars as *E. faecium*, and produces an amplicon characteristic for the genus *Enterococcus.* Strains PKT0003 produced gas from the fermentation of glucose, display carbohydrate fermentation profiles similar to that of *Leuconostoc* species.

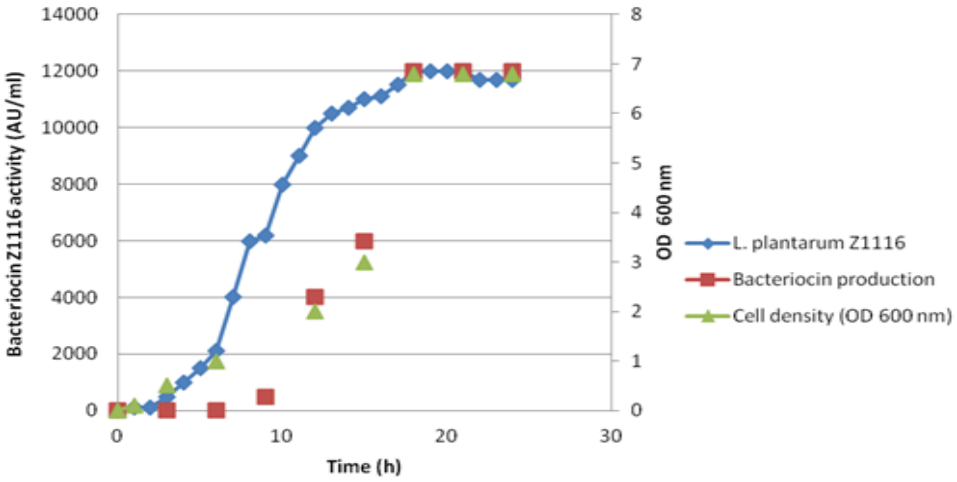

Figure 2. Growth of *L. plantarum* Z1116 in MRS broth in association with bacteriocin production at different cell densities.

Figure 2 shows the growth of *L. plantarum* Z1116 in MRS broth in association with bacteriocin production at different cell densities. This established the activities of bacteriocin Z1116 (500 AU ml^{-1}) at the 9^{th} hour and which progresses gradually till the highest level of activity (12,000 AU ml^{-1}) was attained at the 18^{th} hour of growth in MRS broth ($30^{O}C$). This remained at this level of activity for the duration of fermentation which elapsed at the 24^{th} hour. This high level of activity of bacteriocins occurred at a high cell density (6.8 OD_{600} nm) at the 18^{th} hour of fermentation.

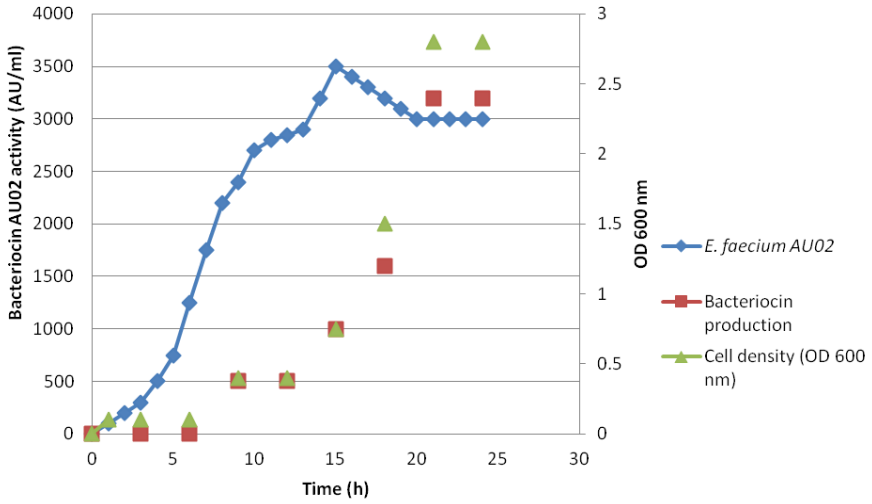

Figure 3. Growth of *E. faecium* AU02 in MRS broth in association with bacteriocin production at different cell densities.

Figure 3 shows the growth of *E. faecium* AU02 in MRS broth in association with bacteriocin activity at varying cell densities. The highest level of bacteriocin AU02 activity (3,200 AU ml^{-1}) was recorded at the 21st hour of growth and this was followed by stable production for remaining 3 hours of fermentation process. The cell density was highest (2.8 OD_{600} nm) at 21st hour of fermentation and which also remained stable for the next 3 hours of the process.

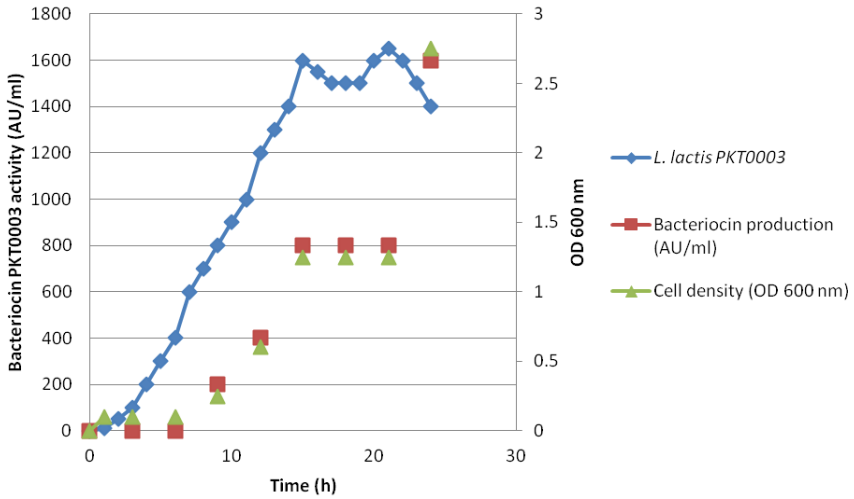

Figure 4. Growth of *Leuconostoc lactis* PKT0003 in MRS broth in association with bacteriocin production at different cell densities.

Figure 4 shows the growth of *Leuconostoc lactis* PKT0003 in MRS broth in association with bacteriocin production at different cell densities. Growth was monitored and recorded and was found that bacteriocin production picked up at the 9th hour (200 AU ml^{-1}) and progresses

over the next 15 hours where the highest level of activity (1600 AU ml^{-1}) was recorded at the 24th hour. At this period of fermentation, cell density measured 2.75 OD_{600} nm.

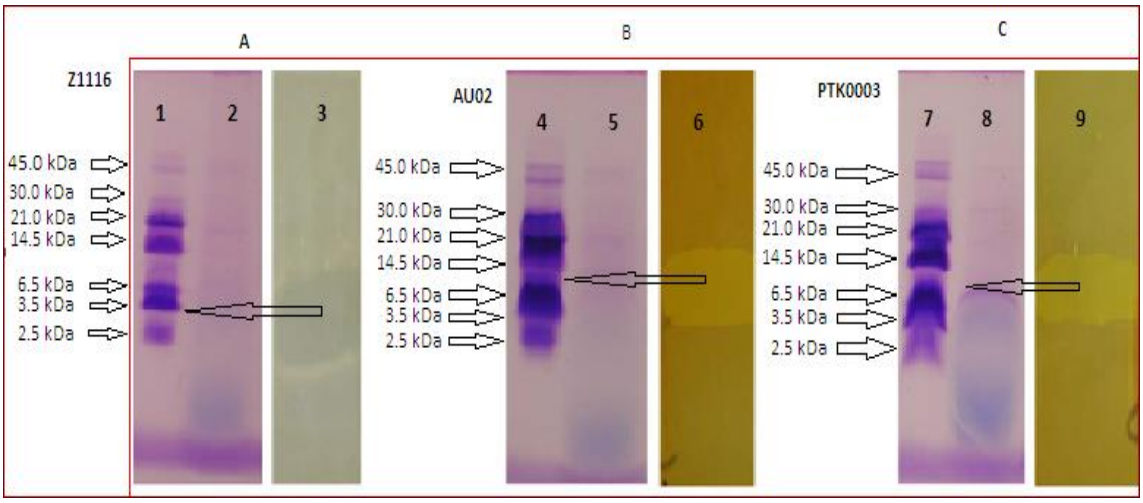

Figure 5: Tricine-SDS–PAGE of bacteriocins Z1116, AU02 and PKT0003. Lanes 1, 4 and 7: molecular mass marker (2.5–45.0 kDa, Amersham). Lanes 2, 5 and 8: peptide bands stained with Coomassie Blue R250. Lanes 3, 6 and 9: zones of growth inhibition corresponding to the position of bacteriocins Z1116, AU02 and PKT0003, respectively. The gel was overlaid with *E. faecium* HKLHS and *L. sakei* DSM 20017, respectively (ca. 1 x 10^6 CFU/ml) suspended in BHI and MRS agar. The sizes of bacteriocins Z1116, AU02 and PKT0003, as revealed by tricine-SDS–PAGE, were 3.2 kDa, 10 kDa and 10 kDa, respectively.

Table 3 shows the factors affecting the antimicrobial activity of bacteriocins Z1116, AU02 and PKT0003. Cell-free supernants of bacteriocins Z1116, AU02 and PKT0003 were subjected to treatments with some enzymes, surfactants, EDTA and varying pH and temperature values. The antimicrobial activity of the three bacteriocins was deactivated after treatment of the cell-free supernatants with proteinase K, papain, pepsin and trypsin. No change in activity levels was recorded when the cell-free supernatants of strains Z1116 and AU02 were treated with α-amylase and catalase. Treatment of the cell-free supernatant containing bacteriocin PKT0003 with α-amylase resulted in loss of antibacterial activity. All three bacteriocins remained active after incubation at pH 2.0–8.0. Treatment at pH 12.0 resulted in the loss of activity for strains AU02 and PKT0003. No decrease in antimicrobial activity was recorded after treatment of the cell-free supernatants at 25, 30, 37, 45 and 60^0C for 60 and 120 min.

Table 3: Factors affecting the antimicrobial activity of bacteriocins Z1116, AU02 and PKT0003

Treatment	Bacteriocins		
	Z1116	AU02	PKT0003
Enzymes (1.0 or 0.1 mg/ml)			
α-Amylase	+	+	-
Catalase	+	+	+
Proteinase K, papain, pepsin, trypsin	-	-	-
Surfactants (1% final concentration)			
SDS, Tween 20, Tween 80, urea, Triton X-100	+	+	+
Triton X-114	-	-	+
Protease inhibitor (1.0, 2.0, 5.0 mm)			
Na-EDTA	+	+	+
Ph			
2.0-8.0	+	+	+
10.0	+	-	+
12.0	+	-	-
Temperature (^{0}C) (1 h):			
25, 30, 37, 45, 60	+	+	+
100	+	+	+
Temperature (^{0}C) (2 h):			
25, 30, 37, 45, 60	+	+	+
100	+	+	-
Temperature (^{0}C) (120 mins):			
121	+	+	-

Keys: - = activities affected; + = activities not affected

Figures 6 and 7 show the effects of bacteriocins produced by A) *Lactobacillus plantarum* strain Z1116, B) *Enterococcus faecium* AU02 and C) *Leuconostoc lactis* PKT0003 on the growth of *E. faecium* HKLHS and *L. sakei* DSM 20017, respectively. The modes of activity of these bacteriocins were bactericidal against *E. faecium* HKLHS and *L. sakei* DSM 20017. Inhibition of growth of test organism was observed at the introduction of only the clinical antibiotic (rifampicin) at final concentration of 0.2 μg ml^{-1} but this was not statistically significant ($P > 0.05$). Rifampicin at final concentrations of 0.1 μg ml^{-1} and 0.05 μg ml^{-1} showed very weak inhibition of growth, and thus, not statistically significant as compared with the control experiment. It was interesting to establish the synergistic effects of rifampicin and these bacteriocins on *L. monocytogenes* NCTC 4885. Rifampicin at final concentration of 0.2 μg ml^{-1} and bacteriocin Z1116 at concentration of 160 AU ml^{-1} exerted bactericidal effect on the test organism while this synergistic effect was also well established at lower concentrations of the antibiotic and bacteriocins.combined (Figure 8).

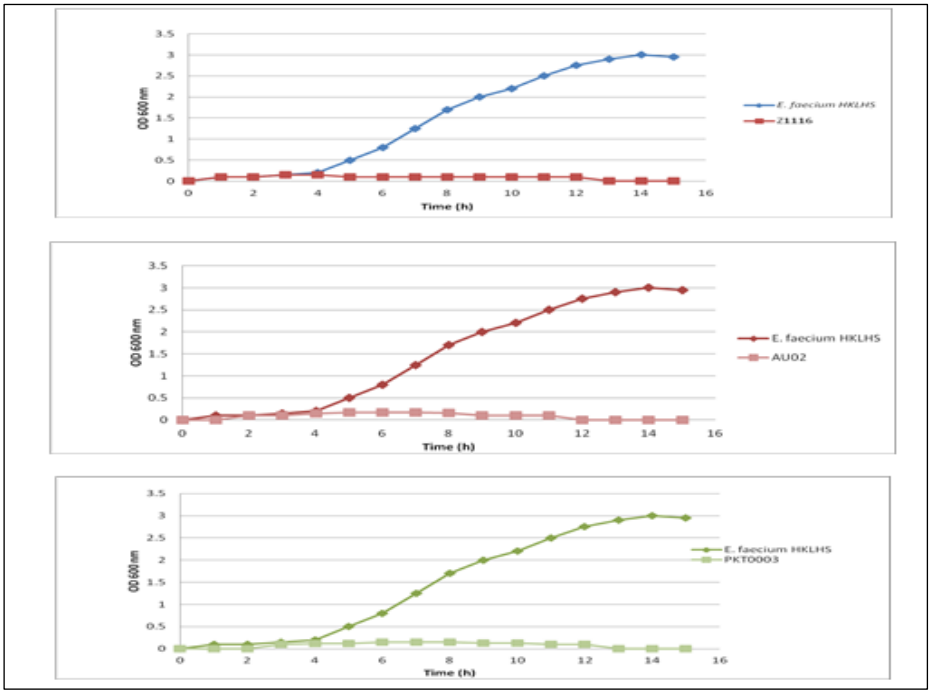

Figure 6: Effect of bacteriocin produced by A) *Lactobacillus plantarum* strain Z1116, B) *Enterococcus faecium* AU02 and C) *Leuconostoc lactis* PKT0003 on the growth of *E. faecium* HKLS.

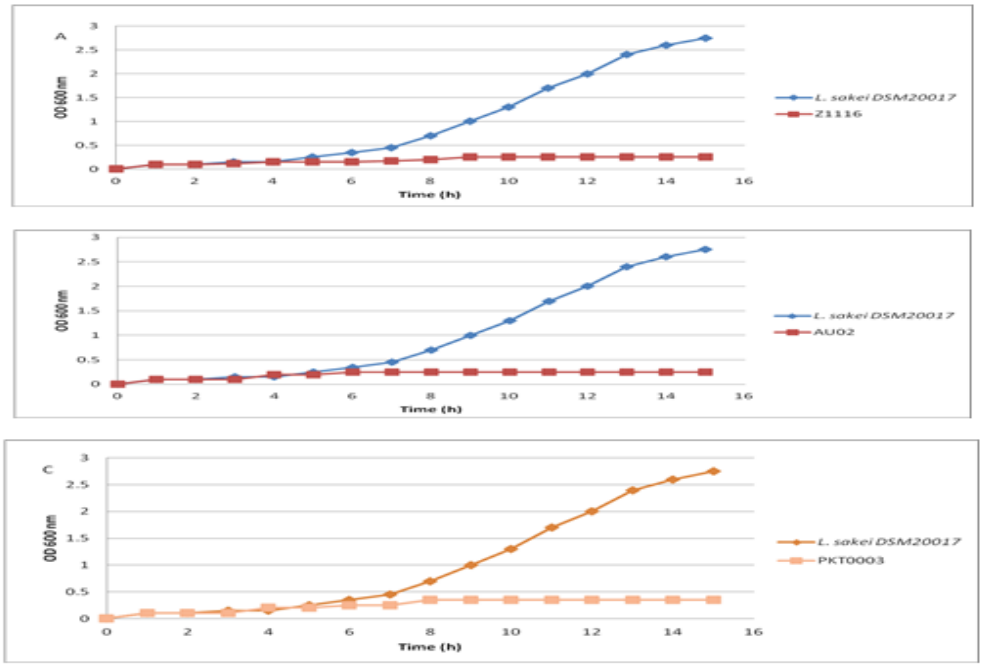

Figure 7: Effect of bacteriocin produced by A) *Lactobacillus plantarum* strain Z1116, B) *Enterococcus faecium* AU02 and C) *Leuconostoc lactis* PKT0003 on the growth of *L. sakei* DSM20017.

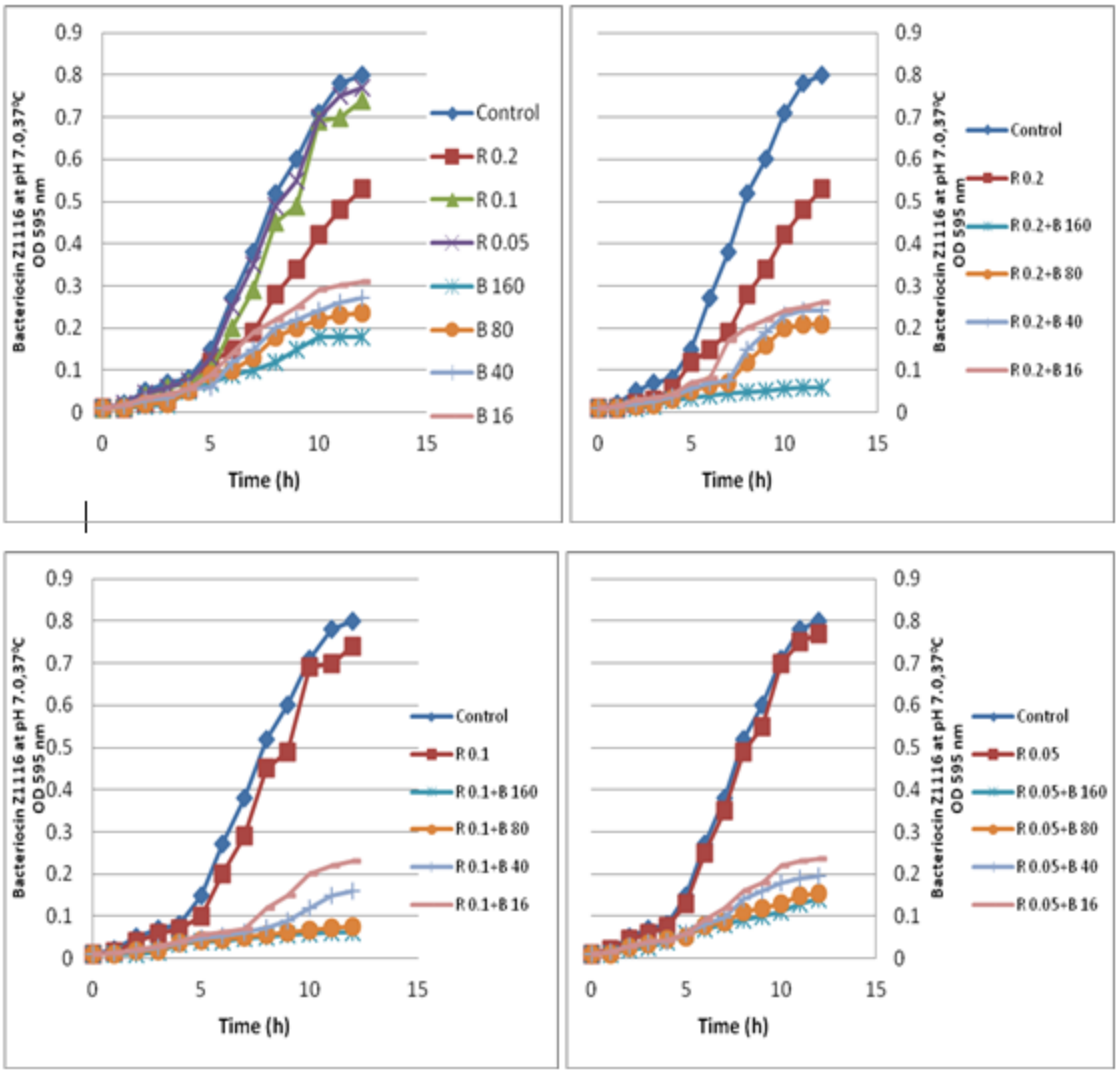

Figure 8: Effect of bacteriocin Z1116 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885.
Control = no bacteriocin or rifampicin added to BHI with corrected to 7.0; R 0.2 = rifampicin at final concentration of 0.2µg ml^{-1}; R 0.1=rifampicin at final concentration of 0.1 µg ml^{-1}; R 0.05=rifampicin at final concentrationof 0.05 µg ml-1; B 160 = bacteriocin Z1116 at concentration of 160 AU ml^{-1}; B 80=bacteriocin Z1116 at concentration of 80 AU ml^{-1}; B 40=bacteriocin Z1116 at concentration of 40 AU ml^{-1}; B 16=bacteriocin Z1116 at concentration 16 AU ml^{-1}

Figure 9 shows the effect of bacteriocin AU02 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885. Bacteriocin AU02 showed weaker antibacterial activity against *L. monocytogenes* NCTC 4885 as compared with bacteriocin Z1116. Bacteriocin AU02 at concentration of 160 AU ml^{-1} exerted visible antibacterial effect, though not statistically significant ($P > 0.05$). Also, inhibitions of growth exerted by rifampicin at all concentrations were not significantly different from the control ($P > 0.05$). There was, however, notable

synergistic effect of this bacteriocin on the test organism when combined with different concentrations of the antibiotic. This difference was statistically significant ($P < 0.05$).

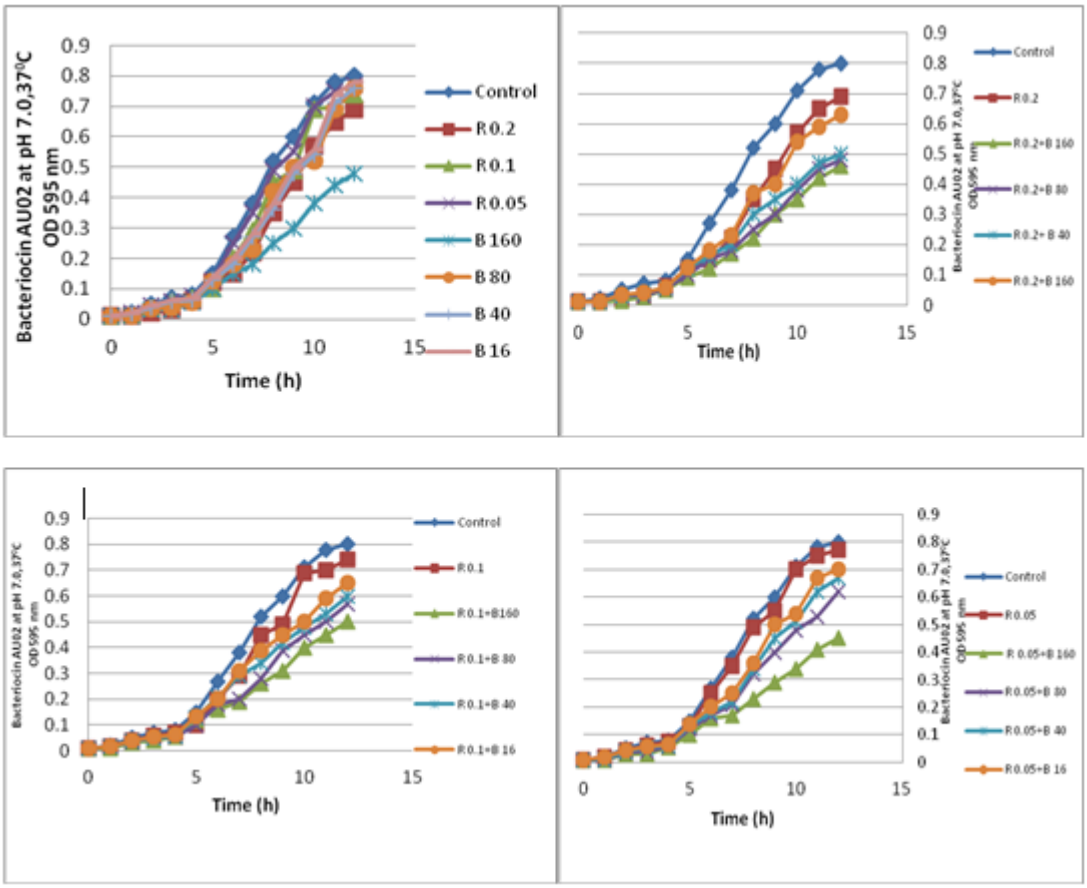

Figure 9: Effect of bacteriocin AU02 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885.

Control = no bacteriocin or rifampicin added to BHI with corrected to 7.0; R 0.2 = rifampicin at final concentration of 0.2ug ml-1; R 0.1=rifampicin at final concentration of 0.1 ug ml-1; R 0.05=rifampicin at final concentrationof 0.05 ug ml-1; B 160 = bacteriocin AU02 at concentration of 160 AU ml-1; B 80=bacteriocin AU02 at concentration of 80 AU ml-1; B 40=bacteriocin AU02 at concentration of 40 AU ml-1; B 16=bacteriocin AU02 at concentration 16 AU ml-1

Figure 10 shows the effect of bacteriocin PKT0003 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885. Just like bacteriocins Z1116 and AU02, bacteriocin PKT0003 exerted stronger antibacterial effect on the test organism than the sub-lethal concentrations of rifampicin investigated in this study. Bacteriocin PKT0003 at concentration of 160 AU ml^{-1} exerted visible antibacterial effect against *L. monocytogenes* NCTC 4885 and this was statistically significant ($P < 0.05$) as compared with control experiment. The synergistic relationship of bacteriocin PKT0003 and rifampicin at their different concentrations was also well established as they were able to exert stronger antimicrobial effects on the test.

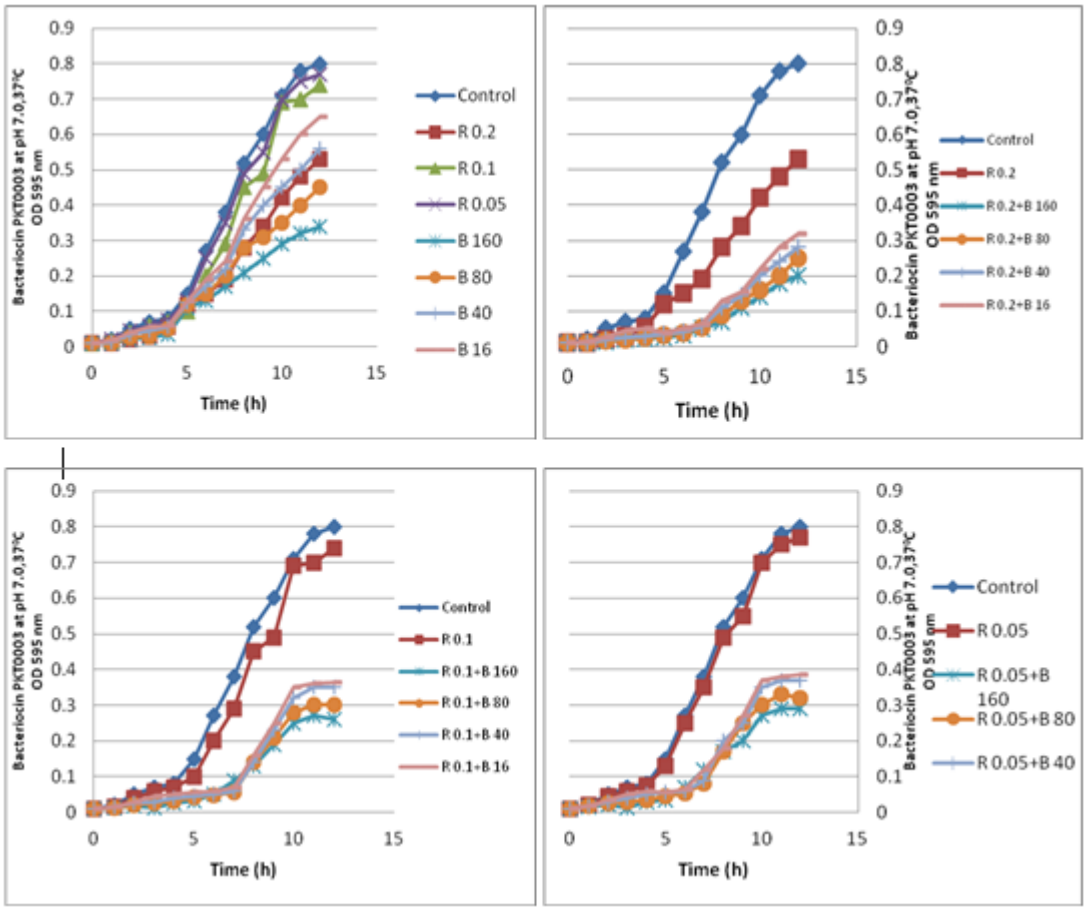

Figure 10: Effect of bacteriocin PKT0003 and antibiotic (rifampicin) on the growth of L. monocytogenes NCTC 4885

Control = no bacteriocin or rifampicin added to BHI with corrected to 7.0; R 0.2 = rifampicin at final concentration of 0.2µg ml^{-1}; R 0.1=rifampicin at final concentration of 0.1 µg ml^{-1}; R 0.05=rifampicin at final concentrationof 0.05 µg ml^{-1}; B 160 = bacteriocin PKT0003 at concentration of 160 AU ml^{-1}; B 80=bacteriocin PKT0003 at concentration of 80 AU ml^{-1}; B 40=bacteriocin PKT0003 at concentration of 40 AU ml^{-1}; B 16=bacteriocin PKT0003 at concentration 16 AU ml^{-1}

Table 4 shows the extracellular levels of β-galactosidase (absorbance detected at 420 nm) recorded after treatment of *E. faecium* HKLHS and *L. sakei* DSM 20017 with bacteriocins Z1116, AU02 and PKT0003. The results obtained about the leakage of DNA, RNA, proteins and β-galactosidase confirmed that bacteriocins Z1116, AU02 and PKT0003 were able to destabilize the permeability of the cell membrane.

Table 4: The extracellular levels of β-galactosidase (absorbance detected at 420 nm) recorded after treatment of *E. faecium* HKLHS and *L. sakei* DSM 20017 with bacteriocins Z1116, AU02 and PKT0003

(A)	Intact cells of *E. faecium* HKLHS	Partially broken cells of *E. faecium* HKLHS	Bacteriocin	*E. faecium* HKLHS treated with bacteriocin
Z1116	0.023	0.172	0.014	0.259
AU02	0.023	0.172	0.090	0.112
PKT0003	0.023	0.172	0.012	0.203

(B)	Intact cells of *L. sakei* DSM 20017	Partially broken cells of *L. sakei* DSM 20017	Bacteriocin	*L. sakei* DSM 20017 treated with bacteriocin
Z1116	0.020	0.168	0.090	0.212
AU02	0.020	0.168	0.017	0.176
PKT0003	0.020	0.168	0.007	0.181

Table 5 shows the percentage adsorption of bacteriocins Z1116, AU02 and PKT0003 to cells of *E. faecium* HKLHS and *L. sakei* DSM 20017. Incubation of the producer cells in the presence of 100 mM NaCl at pH 2.0 did not result into liberation of activity of bacteriocins AU02 and PKT0003, suggesting that these three bacteriocins do not adsorb to cell-surfaces of the producer cells. For bacteriocins Z1116, activity was observed after treatment with 100 mM NaCl at pH 2.0 which suggests the ability of the bacteriocin to adsorb to the cell-surface of the producer cells.

Table 5: Adsorption of bacteriocins Z1116, AU02 and PKT0003 to cells of *E. faecium* HKLHS and *L. sakei* DSM 20017

Bacteriocins	*E. faecium* HKLHS	% Adsorption	*L. sakei* DSM 20017	% Adsorption
Z1116	+	90%	+	90%
AU02	-	20%	-	30%
PKT0003	-	30%	-	30%

Discussion

In this study, bacteriocigenic LAB strains isolated were *Lactobacillus plantarum* Z1116, *Enterococcus faecium* AU02 and *Leuconostoc lactis* PKT0003 and other LAB isolates were *Lactobacillus plantarum, L. fermentum* and *L. pentosus.* The bacteriocins inhibited the growth of *E. faecalis* HKLHS, *Listeria innocua* LMG 13568, *Listeria ivanovii* subsp. *Ivanovii* ATCC 19119, *E. coli* P40, *Klebsiella pneumoniae* P30 and *Pseudomonas* sp. P28. Todorov and Dicks

(2006a) reported similar activity against some Gram-negative bacteria for bacteriocins ST242BZ, ST284BZ, ST414BZ, ST461BZ and ST712BZ produced by *L. paracasei* ST242BZ and ST284BZ, *L. plantarum* ST414BZ, *L. rhamnosus* ST461BZ and *L. pentosus* ST712BZ. More recently, studies on some Afican food condiments showed that *Leuconostoc lactis* T196 from *Colocynthis cintrullus* (Bello *et al*., 2016a) and *Leuconostoc lactis* DZ2 from *Irvingia. gabonensis* (Bello *et al*., 2016b) exerted antibacterial activities against both Gram-positive and Gram-negative bacterial strains.

Strain Z1116 did not produce gas from glucose and fermented carbohydrates reported typical for *L. plantarum*. DNA amplification yielded a fragment identical in size to that reported for *L. plantarum.* Strain AU02 did not produce gas from glucose, fermented the same sugars as *E. faecium*, and produced an amplicon characteristic for the genus *Enterococcus*. Strains PKT0003 produced gas from the fermentation of glucose, displayed carbohydrate fermentation profiles similar to that of *Leuconostoc* spp, and DNA fragments identical in size to that reported for *Leuconostoc lactis.* The activity of bacteriocin Z1116 was detected after 3 h of growth. The activity of bacteriocins AU02 and PKT0003 was detected only after 9 h of growth. This corresponded to the early and middle stationary phase of growth, suggesting that the bacteriocins are secondary metabolites. Similar results were reported for bacteriocins HV219 and A.264 produced by L. *lactis* subsp. *lactis* (Cheigh *et al.*, 2002), and bacteriocin ST15 produced by *E. faecalis* (De Kwaadsteniet *et al.*, 2005).

There were similarities in the sizes of bacteriocins Z1116 (3.2 kDa), AU02 (10.0 kDa) and PKT0003 (10.0 kDa) with other bacteriocins produced by lactic acid bacteria. The sizes recorded for these bacteriocins are within the range reported for most bacteriocins produced by *Lactobacillus* spp and *Enterococcus* spp (De Vuyst and Vandamme, 1994). Bacteriocins T196 and DZ2 were 3.2 kDa and 6.5 kDa, respectively (Bello *et al*., 2016a,b). Bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ are between 2.3 and 3.3 kDa in size (Von Mollendorff *et al.*, 2006). Similar results were reported for bacteriocins ST194BZ (3.0 kDa and 14.0 kDa), ST242BZ (10.0 kDa), ST284BZ (3.5 kDa), ST414BZ (3.7 kDa), ST461BZ (2.8 kDa), ST462BZ (8.0 kDa), ST664BZ (6.5 kDa), ST712BZ (14.0 kDa) (Todorov and Dicks, 2006a). The properties of the three bacteriocins found in this study allow their characterization as group IIa bacteriocins produced by lactic acid bacteria, as they display similar properties in terms of molecular weight, heat and pH stability and sensitivity to proteolytic enzymes (Parente and Ricciardi, 1999; Verschuere *et al*., 2000; Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Drider *et al.*, 2006; Gálvez *et al*., 2007; Mojgani and Amirinia, 2007; Todorov *et al*., 2010; Belguesmia *et al*., 2011).

The antimicrobial activity of the bacteriocins characterized in this study was affected by treatment of the cell-free supernatants with proteinase K, papain, pepsin and trypsin. No change in activity levels was recorded when the cell-free supernatants of strains Z1116 and AU02 were treated with α-amylase and catalase. Treatment of the cell-free supernatant containing bacteriocin PKT0003 with α-amylase resulted in loss of antibacterial activity. All three bacteriocins remained active after incubation at pH 2.0–8.0. Bacteriocin AU02 lost activity after treatment at pH 10.0. Treatment at pH 12.0 resulted in the loss of activity for strains AU02 and PKT0003. No decrease in antimicrobial activity was recorded after treatment of the cell-free supernatants at 25 30, 37, 45 and 60^{O}C for 60 and 120 min. The activity of bacteriocin PKT0003 decreased after 120 min at 100^{O}C.

Surfactants such as SDS, Tween 20, Tween 80, urea and Triton X-100 had no effect on the activity of the three bacteriocins. Addition of Triton X-114 to bacteriocins Z1116 and AU02 led

to decreased activity. Addition of 1.0, 2.0 or 5.0 mM EDTA to the bacteriocins did not affect their activity. Complete inactivation of the bacteriocins was observed after treatment of the cell-free supernatants with proteolytic enzymes, confirming the proteinaceous nature of the antimicrobial compounds. The sensitivity of bacteriocins PKT0003 to 100^{O}C after 120 min and 121^{0}C after 20 min may be a result of their molecular mass (10.0 kDa), although the stability of bacteriocin AU02, a 10.0 kDa peptide was not affected by treatment for 120 min at 100^{O}C. A difference in the structures of bacteriocin AU02 and PKT0003 may be a reason for these results. The Bozacin B.14 was inactivated after 10 min at 90–121^{O}C (Ivanova *et al*., 2000). Not all bacteriocins are heat-stable. For certain bacteriocins (e.g. leucocin F10), pH influences temperature stability. Leucocin F10 is resistant to high temperatures at pH 3.0 and 5.0, but sensitive to the same temperatures at pH 7.0 and 9.0 (Parente *et al.*, 1996).

The mode of activity of bacteriocins Z1116, AU02 and PKT0003 is bactericidal. No growth was recorded when the bacteriocin-treated cells of *E. faecium* HKLHS and *L. sakei* DSM 20017 were plated onto MRS agar. This agrees with the report of Von Mollendorff *et al*. (2006) on bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ, produced by *L. plantarum* JW3BZ and JW6BZ and *L. fermentum* JW11BZ and JW15BZ, respectively, on inhibition of *L. sakei* DSM 20017, implicating that these four bacteriocins had a bactericidal mode of action. Similar result was recorded for pentocin ST18 produced by P. *pentosaceu*s ST18 (Todorov & Dicks, 2005c), mesentericin ST99 produced by *L. mesenteroides* subsp. *dextranicum* ST99 (Todorov & Dicks, 2004), and the African fermented foods B.14 described for L. *lactis* subsp. *lactis* B.14 (Ivanova *et al.*, 2000).

The combined application of the sublethal levels of clinical antibiotic (rifampicin) and the three bacteriocins, antibacterial activity was strongly increased. Results showed significant inhibitions ($P < 0.05$) of the growth of *L. monocytogenes* NCTC 4885 when bacteriocin Z1116 at final concentrations of 160 AU ml^{-1}, 80 AU ml^{-1}, 40 AU ml^{-1} and 16 AU ml^{-1} were added to BHI medium with pH corrected to 7.0. Inhibition of growth of test organism was also observed at the introduction of only the clinical antibiotic (rifampicin) at final concentration of 0.2 μg ml^{-1} but this was not statistically significant ($P > 0.05$).

Rifampicin at final concentrations of 0.1 μg ml^{-1} and 0.05 μg ml^{-1} showed very weak inhibition of growth, and thus, not statistically significant as compared with the control experiment. Rifampicin at final concentration of 0.2 μg ml^{-1} and bacteriocin Z1116 at concentration of 160 AU ml^{-1} exerted bactericidal effect on the test organism while this synergistic effect was also well established at lower concentrations of the antibiotic and bacteriocins.combined. These results indicate that the mechanism by which the cationic peptide increases the effectiveness of these antibiotics is through the dissipation of the proton gradient responsible for the extrusion of these compounds. This synergism between antibiotics, particularly rifampicin investigated in this study, and bacteriocins are important in order to reduce the level of the MIC of the antibiotics. Similar synergetic effects may be an important step in the future treatment of multidrug resistant strains.

Inhibitions of growth exerted by rifampicin at all concentrations were not significantly different from the control ($P > 0.05$). There was, however, notable synergistic effect of this bacteriocin on the test organism when combined with different concentrations of the antibiotic. This difference was statistically significant ($P < 0.05$). Bacteriocin PKT0003 at concentration of 160 AU ml^{-1} exerted visible antibacterial effect against *L. monocytogenes* NCTC 4885 and this was statistically significant ($P < 0.05$) as compared with control experiment. The synergistic relationship of bacteriocin PKT0003 and rifampicin at their different concentrations was also

well established as they were able to exert stronger antimicrobial effects on the test organism than when used unilaterally.

In a similar study by Minahk *et al*. (2004), the effect of sub-lethal concentrations of enterocin CRL35, a cationic peptide, on the activity of erythromycin, chloramphenicol and tetracycline was studied. At studied sub-lethal concentrations, the peptide induces a significant membrane gradient dissipation without appreciable cell death. A plausible explanation is that membrane depolarization is necessary but not sufficient to produce cell death, and another concentration dependent step, not described at present may be implicated. It was reported that pleurocidin and its derivatives which are antimicrobial peptides from eukaryotic organisms lost their ability to damage cell membranes at sub-lethal concentrations, whilst maintaining their capacities to inhibit macromolecular synthesis (Patrzykat *et al*., 2002).

The leakage of DNA, RNA, proteins and β-galactosidase confirmed that bacteriocins Z1116, AU02 and PKT0003 destabilized the permeability of the cell membrane. Similar results have been reported for buchnericin LB (Yildirim *et al.*, 1999), plantaricin 423 (Todorov and Dicks, 2006b), pediocin AcH (Bhunia *et al.*, 1991) and bacteriocin HV219 (Todorov *et al.*, 2007). Incubation of the producer cells in the presence of 100 mM NaCl at pH 2.0 did not result into detection of activity of bacteriocins AU02 and PKT0003, suggesting that these bacteriocins do not adsorb to cell-surfaces of the producer cells. Similar results were reported for bacteriocins ST194BZ, ST242BZ, ST284BZ, ST414BZ, ST461BZ, ST462BZ, ST664BZ and ST712BZ (Todorov & Dicks, 2006a).

Von Mollendorff *et al.* (2006) reported a similar result that bacteriocins JW3BZ, JW11BZ and JW15BZ retained their activity after treatment with 100 mM NaCl at pH 2.0, indicating that bacteriocins JW3BZ, JW11BZ and JW15BZ adsorb to the cell-surface of the producer strains. This is in contrast with the result of Jillian (2006) who reported the adsorption of BacST8KF to sensitive and resistant strain of Gram-positive bacteria with percentage adsorption ranging from 20% for *Lb plantarum* LMG 13556 to 80% for *Lb casei* LHS. The author suggested that the adsorption of BacST8KF to target strains does not confirm the activity of the peptide against the target strain. This was also buttressed by the report of Yildrim *et al.* (2002) where 100% adsorption of buhnericin LB to a strain of *Pediococcus cerevisiae* was insensitive.

Some bacteriocins were found to adhere to the cell-surface of the producers such as pediocin AcH, nisin, sakacin A, leuconocin Lcm by Yang *et al.* (1992). In the case of plantaricin C19, produced by *L. plantarum* C19, maximal adsorption to the producer cells was recorded between pH 5.0 and 7.0, with a complete loss of adsorption at pH 1.5 and 2.0 (Atrih *et al.*, 2001). This phenomenon may be used successfully in the purification of the bacteriocins.

This study revealed that *P. biglobosa* is a rich source of bacteriocigenic LAB, and these bacteriocins and their biosynthetic mechanisms could be useful in food safety and security, preservation, peptide design, infection control and pharmacotherapy. This should help in the control of undesirable bacteria and in designing more powerful and more selective antimicrobial peptides.

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