Molecular screening of bacteriocin produced by lactic acid bacteria from *Irvingia gabonensis* seeds

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Abstract: Food safety has become an increasingly important international concern, the application of antimicrobial peptides from lactic acid bacteria (LAB) that target food pathogens without toxic or other adverse effects should receive greater attention. This work investigated bacteriocin production in LAB from *Irvingia gabonensis* with the characterization of putative bacteriocins.One hundred samples of the food were purchased, ground, serially diluted and cultured on Man-Rogosa-Sharpe agar for the isolation LAB, respectively, using the spread plate technique. Bacteriocin-producing LAB were identified using standard procedures; bacteriocin's activities, and synergetic effect of bacteriocin and rifampicin were measured by spectrophotometric analysis. Student t-test and analyses of variance were employed for analyses of data. *Leuconostoc lactis* DZ2 was the bacteriocigenic LAB isolate; these inhibited the growth of standard organisms. Other LAB isolates were *Lactobacillus rhamnosus, Lactobacillus pentosus* and *Leuconostoc lactis.* The synergistic effects of characterized bacteriocin and rifampicin tested on organisms showed significant differences ($P < 0.05$). This study revealed that *I. gabonensis* contained bacteriocin-producing LAB that could be used as bio-preservatives. It suggested that bacteriocins serve as alternatives to classical antibiotics in treating bacterial infections, and their roles in food safety cannot be over-emphasized.
[Olorunjuwon O. Bello, Samuel A. Bankole, Olubukola O. Babalola. **Molecular screening of bacteriocin produced by lactic acid bacteria from *Irvingia gabonensis* seeds.** *Nat Sci* 2016;14(8):67-78]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 11. doi:10.7537/marsnsj14081611.

Keywords: Bacteriocin; *Irvingia gabonensis*; lactic acid bacteria; bio-preservatives; food safety.

1. Introduction

Irvingia is a genus of African and Southeast Asian trees in the family *Irvingiaceae*. It is a dominant tropical forest tree of West and Central Africa, which is rated as one of the most domestically consumed wild fruit tree (Ladipo *et al*., 1995). In Africa, particularly in the Southern and Eastern regions of Nigeria, *Irvingia gabonensis*, sometimes called bush mango, African mango seed, dika nut or *apon*, is grown and consumed basically because of its kernels which serve as a major condiment in the preparation of Nigeria's famous '*ogbona*' or "*apon*" soup. They bear edible mango-like fruits, and are especially valued for their fat- and protein-rich nuts. The fruit is a large drupe, with fibrous flesh. *I. gabonensis* is highly valued for its health and medicinal benefits and agricultural potentials (Etebu, 2012). Studies have shown that seed extract of *I. gabonensis* caused a significant reduction in body weight among obese people in Cameroon (Ngondi *et al*., 2005).

The demand by consumers for a decrease in the use of chemical additives in food has led to research on the use of natural antimicrobial substances secreted by food fermentative bacteria to inhibit undesirable bacteria. Bacteriocins are ribosomally synthesized polypeptides with activity against genetically closely related bacteria and other strains. The peptides generally vary with regards to their mode of action, molecular weight, genetic origin, biochemical properties and spectrum of activity (Bello *et al*., 2016).

Lactic acid bacteria (LAB) are a diverse and very useful group of bacteria that, while not adhering to a strict taxonomic group, are gathered on the basis of shared properties (Oguntoyinbo and Narbad, 2012) and have the common trait of producing lactic acid (LA) as a major or sole fermentation product. For these reasons, LAB have historically been associated with the fermentation of foods, and as a result many LAB, are generally recognized as safe (GRAS) and/or probiotics (Mayo *et al*., 2010). Bacteriocins produced by LAB display a high degree of heterogeneity. Bacteriocins have attracted much interest as those with GRAS status are safe for human consumption and can be used in the preservation of food products, without any implication on consumer health (Macwana and Muriana, 2012).

The desirable property of a probiotic strain is the ability to produce antimicrobial substances such as bacteriocins that offer the potential to provide an advantage in competition and colonization of the gastrointestinal tract. Bacteriocins are peptides produced by bacteria that inhibit or kill other related and unrelated microorganisms. Bacteriocin was first identified by Gratia (1925) as an antimicrobial protein produced by *Escherichia coli* and named colicin

(Collins *et al*., 2010). 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 (Bello *et al*., 2016). Thus, because of consumer demand for higher quality and natural foods, as well as of strict government requirements to guarantee food safety, food producers have faced conflicting challenges (Franz, 2010).

Regardless of the strain and bacteriocin in question, it is fair to say that the application of bacteriocin-producing LAB, alone or in combination with additional antimicrobial hurdles, continues to be a relatively under-utilized strategy that, through various enhancements, such as the information in this study, could be more widely applied by the food industry. This work aims to identify bacteriocin-producing LAB in seeds of *Irvingia gabonensis* and characterize putative bacteriocin.

2. Material and Methods

2.1 Sources, processing and bacteriological analyses of samples

One hundred seeds of *Irvingia gabonensis* were purchased over a six-month period from local sellers from different markets in southwestern Nigeria. Samples were usually kept overnight in the refrigerator at 4 ^{O}C and transported to the laboratory in sterile bags packed in insulated containers with ice packs. Bacteriological analyses were usually carried out within 24 h after sampling in the Microbiology Laboratory, Department of Microbiology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria.

2.2 Isolation of lactic acid bacteria and screening for bacteriocin-producing isolate

Samples 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^{0}C. Colonies with inhibition zones were selected, cultured in MRS broth (Biolab) and tested for antimicrobial activity against *E. faecium* HKLHS and *L. sakei* DSM 20017 by using the agar-spot test and disc diffusion methods (Todorov and 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 (Todorov and Dicks, 2005a; Todorov, 2010; Bello *et al*., 2016).

2.3 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 by Stiles and Holzapfel (Stiles and Holzapfel, 1997). Carbohydrate fermentation reactions were recorded by using the API 50 CHL and API 20 Strep test strips (Biomérieux, Marcy-l'Etiole, France). Results obtained were compared with carbohydrate fermentation reactions listed in *Bergey's Manual of Systematic Bacteriology* (Felske *et al*., 1997). Further identification was by genus and species-specific primers. A O'GeneRuler™ 100-bp DNA Ladder (Fermentas, USA), O'GeneRuler™ 1 kb DNA Ladder (Fermentas) and O'GeneRuler™ Ultra Low Range DNA Ladder (Fermentas) were used as molecular markers. 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′). 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).

Amplification was done in a DNA thermal cycler (GeneSysetm$^{\infty}$ PCR System C1000 Touch Thermal Cycler, Singapore) as follows: 45 cycles of 1 min per cycle at 94^{0}C, and 1 min at 36 ^{O}C, followed by an increase to 72 ^{0}C over 2 min. Extension of the amplified product was at 72^{0}C for 5 min. The amplified products were separated by electrophoresis in 1.4% (w/v) agarose gels in 1x TAE buffer at 100 V for 1 h. Gels were stained in TAE buffer containing 0.5 µg ml^{-1} ethidium bromide (Sigma Diagnostics, St. Louis, MO, USA). Banding patterns were analysed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).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 ^{O}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.) (Bello *et al*., 2016).

2.4 Bacteriocin production

MRS broth (Biolab) was inoculated with a 24-h-old culture (2%, v/v) of a bacteriocin-producing strain of *Leuconostoc lactis* DZ2. Incubation was at 30 ^{O}C, without agitation. 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 (containing both Gram-positive and Gram-negative) 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 (Todorov, 1997; Bello *et al*., 2016).

2.5 Molecular size of the bacteriocins

Twenty-four-h-old 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 bacteriocin, cell-free supernatant was treated for 10 min at 80^{0}C. Ammonium sulfate was added slowly to the cell-free supernatant to 80% for *Leuconostoc lactis* T196, stirred for 4 h at 4^{0}C and then centrifuged (10,000g, 1 h, 4 ^{O}C). The amount of ammonium sulfate was previously optimized for the precipitation of the studied bacteriocin. 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 (1997). 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 Van Reenen *et al.* (1996). *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 (Bello *et al*., 2016).

2.6 Determination of pH

pH was measured using digital pH meter as described by Bello *et al.* (2016)

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

Cell-free supernatant of bacteriocin-producing strain, obtained by centrifugation (8000g, 10 min, 4 ^{O} C), were adjusted to pH 6.0 with 1 M NaOH. Two mils (2 mls) 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 bacteriocin in cell-free supernatants was determined as described by Todorov and Dicks (2006a) and Todorov (2010). 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 (Todorov *et al*., 2006). 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*., 2006a; Todorov, 2010).

2.7 Response of bacteria to bacteriocin

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 bacteriocin T196, 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^{0}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. All experiments were done in duplicate in two independent occasions (Todorov *et al*., 2006a; Bello *et al*., 2010).

2.8 Adsorption study of the bacteriocin to the producer cells

The ability of a bacteriocin to adsorb to producer cells was studied according to the method described by Yang, Johnson and Ray (1992). After 18 h of growth at 30^{0}C, the culture was adjusted to pH 6.0, the

cells were harvested (10,000g, 15 min, 4^0C) 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 described earlier. The percentage adsorption of bacteriocin 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, 2011)

2.9 Statistical analysis of data obtained

The data generated was subjected to statistical analyses. Paired-Samples T-test and One-way Analysis of Variance (ANOVA) were employed to establish the difference in the microbial activity of bacteriocin and antibiotic (rifampicin) against *L. monocytogens* NCTC 4885 using SPSS version 17.0.

3. Results

Table 1 shows the bacteriocigenic LAB and other LAB isolates from protein-rich foods from *Irvingia gabonensis*. *Leuconostoc lactis* DZ2 was a bacteriocigenic strain isolated from *I. gabonensis*. Other LAB isolates were *Lactobacillus rhamnosus, Lactobacillus pentosus* and *Leuconostoc lactis* were isolated from *I. gabonensis*.

Table 2 shows the spectra of activities of *L. lactis* DZ2 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, 15 (33.33%) were positive to *L. lactis* DZ2.

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, T196 and DZ2 produce gas from the fermentation of glucose, display carbohydrate fermentation profiles similar to that of *Leuconostoc* species.

Figure 2 shows the growth of *L. lactis* DZ2 as associated with bacteriocin level of activity at different cell densities. There was optimum bacteriocin activity (6500 AU ml^{-1}) at the 15th hour followed by reduction to 3100 AU ml^{-1} during the next 9 hours of fermentation process. The highest cell density measured 2.3 OD_{600} nm. Figure 3 shows the tricine-SDS–PAGE of bacteriocins DZ2. The size of bacteriocin DZ2 was 6.5 kDa.

Table 3 shows the factors affecting the antimicrobial activity of bacteriocin DZ2. Cell-free supernant of bacteriocin DZ2 was subjected to treatments with some enzymes, surfactants, EDTA and varying pH and temperature values. The antimicrobial activity of the bacteriocin was inhibited 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 supernatant of the bacterial strain was treated with α-amylase and catalase. The bacteriocin remained active after incubation at pH 2.0–8.0. No decrease in antimicrobial activity was recorded after treatment of the cell-free supernatant at 25 30, 37, 45 and 60 ^{O}C for 60 and 120 min. A decrease in bacteriocin DZ2 activity was recorded after 60 and 120 min at 100 ^{O}C.

Figure 6 shows the effect of bacteriocin DZ2 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885. The antibacterial activity exerted by bacteriocin DZ2 at concentration of 160 AU ml^{-1} was similar to that of rifampicin at the concentration of 0.2 µg ml^{-1} and showed no significant difference from the control ($P > 0.05$)(Figure 6). There exist a good synergistic relationship between the bacteriocin and rifampicin at all concentrations as stronger antibacterial effect was mounted against *L. monocytogenes* NCTC 4885 when the two agents were combined.

Figures 4 and 5 show the effects of bacteriocin produced by *L. lactis* DZ2 on the growth of *E. faecium* HKLHS and *L. sakei* DSM 20017, respectively. The mode of activity of the bacteriocin was bactericidal, as determined against *E. faecium* HKLHS and *L. sakei* DSM 20017.

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 bacteriocin DZ2. The results obtained about the leakage of DNA, RNA, proteins and β-galactosidase confirm that the bacteriocin destabilized the permeability of the cell membrane.

Table 1: Bacteriocigenic LAB and other LAB isolates from *Irvingia gabonensis*

Food product	Bacteriocigenic LAB isolates	Other LAB isolates
Irvingia gabonensis	*Leuconostoc lactis* DZ2	*Lactobacillus rhamnosus* *Lactobacillus pentosus* *Leuconostoc lactis*

Table 2: Antibacterial spectrum of activity of bacteriocins produced by *L. lactis* DZ2

Bacterial Strain	Medium	Temperature (^{O}C)	*L. lactis* DZ2
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. ivanovii subsp. *ivanovii* ATCC 19119[b]	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	+
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.

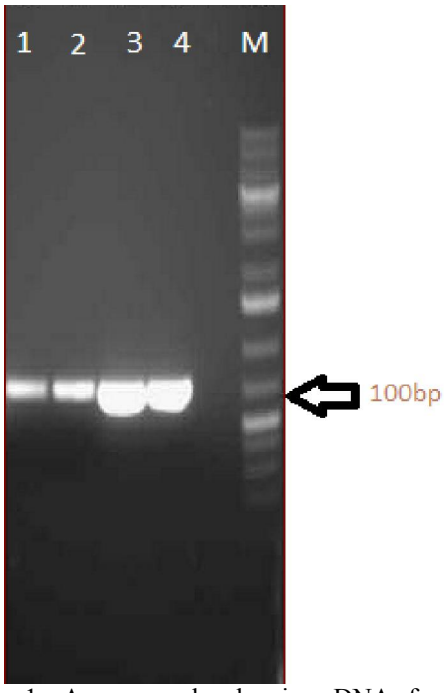

Figure 1: Agarose gels showing DNA fragments obtained after PCR with species-specific and genus-specific primers. Lanes 1: strain DZ2, line 2: strain DZ2, lane 3: *L. lactis* NCDO 533 (DSM 20202T), lane 4: *L. lactis* NCDO 533 (DSM 20202T), lane 4: no DNA loaded and lane M: O'GeneRuler™ 1 kb DNA Ladder (Fermentas).

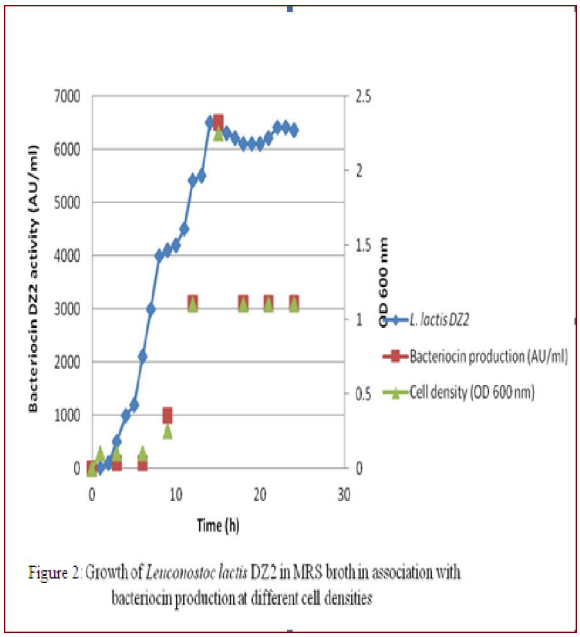

Figure 2: Growth of *Leuconostoc lactis* DZ2 in MRS broth in association with bacteriocin production at different cell densities

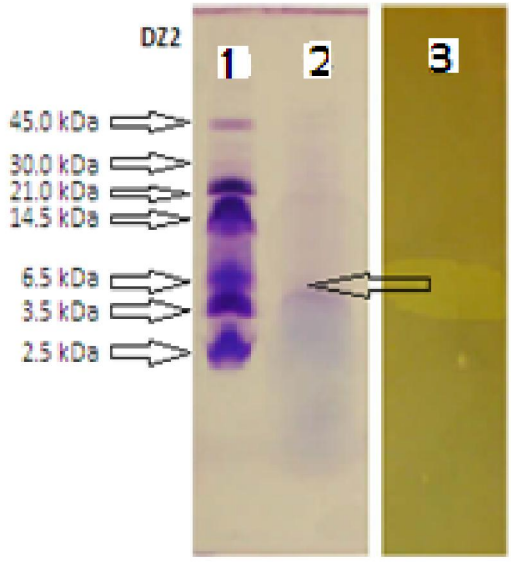

Figure 3: Tricine-SDS–PAGE of bacteriocin DZ2. Lane 1: molecular mass marker (2.5–45.0 kDa, Amersham). Lane 2: peptide bands stained with Coomassie Blue R250. Lane 3: zones of growth inhibition corresponding to the position of bacteriocin DZ2. The gel was overlaid with *E. faecium* HKLHS and *L. sakei* DSM 20017, respectively (ca. 1 x 10^6 CFU/ml) suspended in BHI and MRS agar.

Table 3: Factors affecting the antimicrobial activity of bacteriocin DZ2

Treatment	Bacteriocins
	DZ2
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

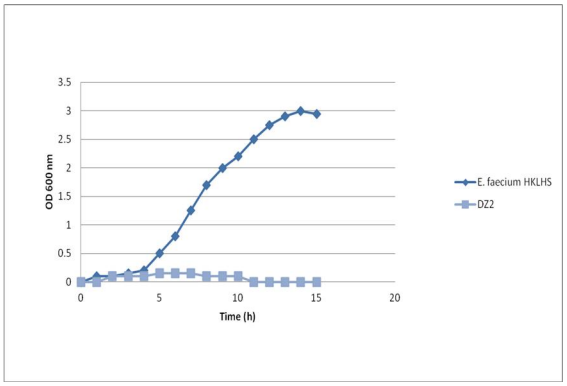

Figure 4: Effect of bacteriocin produced by *Leuconostoc lactis* DZ2 on the growth of *E. faecium* HKLS

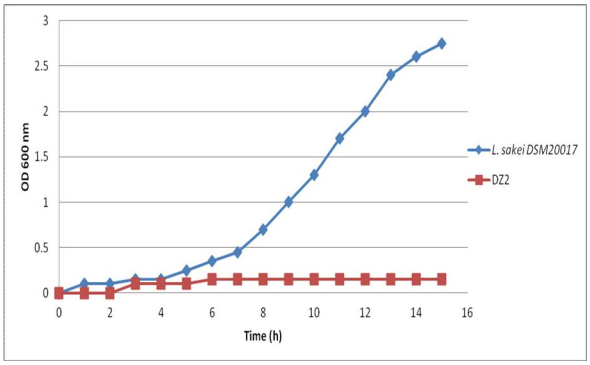

Figure 5: Effect of bacteriocin produced by *Leuconostoc lactis* DZ2 on the growth of *L. sakei* DSM20017

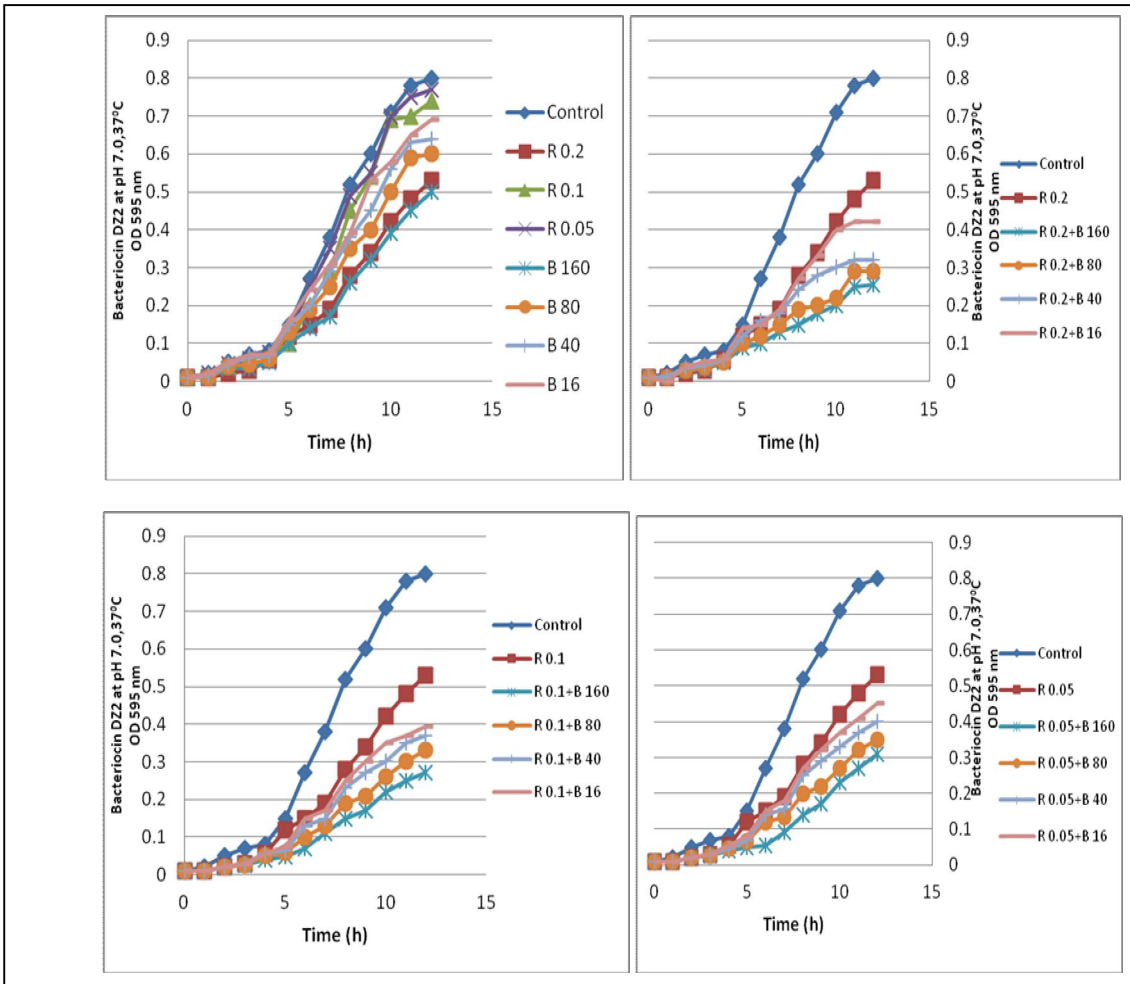

Figure 6: Effect of bacteriocin DZ2 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885. Control = no bacteriocin or rifampicin added to BHI with corrected to 7.0; R 0.2 = rifampicin at final concentration of 0.2µg ml^{-1}; R 0.1=rifampicin at final concentration of 0.1 µg ml^{-1}; R 0.05=rifampicin at final concentrationof 0.05 µg ml^{-1}; B 160 = bacteriocin DZ2 at concentration of 160 AU ml^{-1}; B 80=bacteriocin DZ2 at concentration of 80 AU ml^{-1}; B 40=bacteriocin DZ2 at concentration of 40 AU ml^{-1}; B 16=bacteriocin DZ2 at concentration 16 AU ml^{-1}

Figure 7 shows the percentage adsorption of bacteriocin DZ2 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 DZ2, suggesting that the bacteriocin did not adsorb to cell-surfaces of the producer cells.

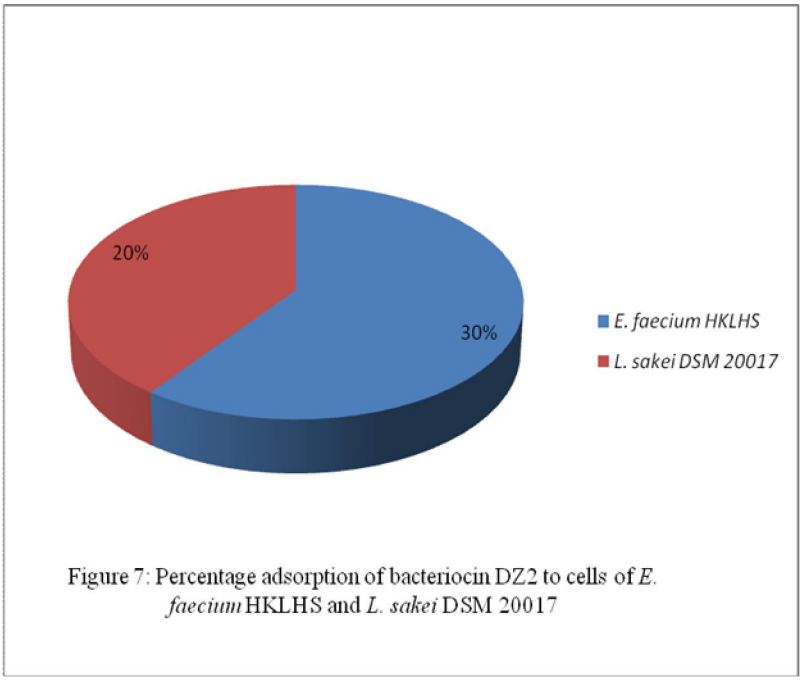

Figure 7: Percentage adsorption of bacteriocin DZ2 to cells of *E. faecium* HKLHS and *L. sakei* DSM 20017

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 bacteriocin DZ2

(A)	Intact cells of *E. faecium* HKLHS	Partially broken cells of *E. faecium* HKLHS	Bacteriocin	*E. faecium* HKLHS treated with bacteriocin
DZ2	0.023	0.172	0.096	0.143
(B)	Intact cells of *L. sakei* DSM 20017	Partially broken cells of *L. sakei* DSM 20017	Bacteriocin	*L. sakei* DSM 20017 treated with bacteriocin
DZ2	0.020	0.168	0.019	0.207

4. Discussion

One bacteriocigenic LAB isolate - *Leuconostoc lactis* DZ2 - was obtained from *I. gabonensis*. Other LAB isolates were *Lactobacillus rhamnosus, Lactobacillus pentosus* and *Leuconostoc lactis* sub *lactis*. The bacteriocigenic strain 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. Bacteriocins of lactic acid bacteria are by definition active against Gram-positive bacteria and usually against species related to the producer strain (De Vuyst and Vandamme, 1994). More recent reports on bacteriocins from LAB with activity against a broad range of Gram-positive and Gram-negative bacteria, e.g. *K. pneumoniae*, *E. coli* and *Pseudomonas* spp (Todorov S.D., Dicks, 2005a) and v*Iru*ses, e.g. herpes simplex v*Iru*s and influenza (Wachsman *et al*., 2003) have been reported.

Few bacteriocins produced by LAB isolated from a cereal-based fermented beverage, boza, with activity against Gram-negative bacteria have been reported. Bacteriocin JW15BZ, produced by *L. fermentum* JW15BZ (Todorov *et al*., 2005; Von Mollendorff *et al*., 2006) is active against *K. pneumoniae* and bacteriocin Bozacin B.14, produced by *L. lactis* subsp. *lactis* 14 (Ivanova *et al*., 2000), against *E. coli.* Similar results about the activity against some Gram-negative bacteria have been reported 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 (Todorov and Dicks, 2006a). The activity of the bacteriocin was detected after 3 h of growth. This corresponded to the early and middle stationary phase of growth, suggesting that bacteriocins are secondary metabolites.

Similar results were reported for bacteriocins HV219 and A.264 produced by L. *lactis* subsp. *lactis*

(Todorov *et al*., 2006a; Cheigh *et al*., 2002). The decrease recorded in the production of bacteriocin DZ2 could be due to the instability of the bacteriocins at low pH, proteolytic degradation, protein aggregation, adsorption to the cell-surface or feedback regulation. Similar results have been recorded for bacteriocins ST13BR and 423 produced by *L. plantarum* (Verellen *et al*., 1998; Todorov *et al*., 2004) and pentocin TV35b described for *L. pentosus* (Okkers *et al*., 1999). Stability of the bacteriocin at pH 2.0–8.0 suggested that activity may not be affected by pH changes during growth.

The size of bacteriocin DZ2 (6.5 kDa) is similar to that described for other bacteriocins produced by lactic acid bacteria. 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 size recorded for DZ) is within the range reported for most bacteriocins produced by *Lactobacillus* spp and *Enterococcus* spp (De Vuyst and Vandamme, 1994; Bello *et al*., 2016).

The properties of the bacteriocin found in this study allow its 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). Characteristics unifying all members of class IIa bacteriocins are (i) they are below or equal to 10 kDa (ii) their pronounced activity against *Listeria* spp, (iii) their resistance to elevated temperatures and pH value, and (iv) their cystibiotic feature attributed to the presence of at least one disulphide bridge, which is crucial for antibacterial activity (Cotter *et al*., 2005; Drider *et al*., 2006; Gálvez *et al*., 2007; Mojgani *et al*., 2007; Bhunia *et al*., 1991; Todorov *et al*., 2007; Todorov *et al*., 2010; Belguesmia *et al*., 2011).

Class IIa bacteriocins were formerly considered as "narrow-spectrum" antibiotics, with antimicrobial activity directed against related strains. However, recently, some class IIa bacteriocins, such as bacteriocin OR-7, enterocin E50-52, and enterocin E760, have been shown also to be active against both Gram-negative and Gram-positive bacteria, including *Campylobacter jejuni*, *Yersinia* spp, *Salmonella* spp, *E. coli* O157:H7, *Shigella dysenteriae*, *S. aureus*, and *Listeria* spp (Bhunia *et al*., 1991; Cotter *et al*., 2005; Drider *et al*., 2006; Belguesmia *et al*., 2011; Pinto *et al*., 2009). This buttresses the broad spectra of activities exhibited by bacteriocins characterized in this study.

The antimicrobial activity of bacteriocin DZ2 was inhibited 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 supernatant of strain DZ2 was treated with amylase and catalase. 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. A decrease in bacteriocin DZ2 activity was recorded after 60 and 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 bacteriocin DZ2. Addition of 1.0, 2.0 or 5.0 mM EDTA (final concentrations) to the bacteriocin did not affect their activity. Complete inactivation of the bacteriocin was observed after treatment of the cell-free supernatant with proteolytic enzymes, confirming the proteinaceous nature of the antimicrobial compounds.

Treatment of cell-free supernatants of bactericigenic strain with catalase and α-amylase did not result in activity changes, except suggesting that the inhibition recorded was not hydrogen peroxide and that carbohydrate moieties were not required for antimicrobial activity. Stability of the bacteriocin DZ2 in the presence of α-amylase is not unusual as similar results have also been reported for other bacteriocins. Leuconocin S (Keppler *et al*., 1994) and carnocin 54 (Lewus *et al*., 1994) are sensitive to α-amylase, suggesting that their activity is associated with glycosylation of the active peptide. Thermostability at 100^{0}C has also been reported for most other bacteriocins (Todorov and Dicks, 2006a; Von Mollendorff *et al*., 2006). The sensitivity of bacteriocin DZ2 to 100 ^{O}C after 120 min and 121 ^{O}C after 20 min may be as a result of its molecular mass (6.5 kDa). The Bozacin B.14 was inactivated after 10 min at 90–121 ^{O}C [24]. Not all bacteriocins are heat-stable. For certain bacteriocins (e.g. leucocin F10), pH influences temperature stability (Parente, Moles and Ricciardi, 1996). 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).

An 87.5% loss in activity was observed for lactocin NK24 after 30 min at 100^{0}C, while it was completely inactivated after 15 min at 121^{0}C (Lee and Paik, 2001). No change in activity was recorded after treatment of cell-free supernatants containing bacteriocins JW3BZ, JW6BZ, JW11BZ and JW15BZ with SDS, urea, Tween 20, Tween 80 and EDTA (Von Mollendorff *et al*., 2006). Treatment of bacteriocins JW3BZ and JW11BZ with Triton X-100 resulted in a loss of activity. Treatment with Triton X-114 destroyed the activity of bacteriocins JW3BZ, JW6BZ and JW11BZ (Von Mollendorff *et al*., 2006). Similar

results were reported for pediocin ST18 (Todorov and Dick, 2005c), enterocin EJ97 (Gálvez *et al*., 2007), the Bozacin B.14 (Ivanova *et al*., 2000), and bacteriocins isolated from a cereal-based food reported by Todorov and Dicks (2006a).

The mode of activity of the bacteriocin is bactericidal, as determined against *E. faecium* HKLHS and *L. sakei* DSM 20017. No growth was recorded when the bacteriocin-treated cells of *E. faecium* HKLHS and *L. sakei* DSM 20017 were plated onto MRS agar. Similar results were recorded for 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 (Von Mollendorff *et al*., 2006). As well, similar result was recorded for pentocin ST18 produced by P. *pentosaceu*s ST18 (Todorov and Dick, 2005c), mesentericin ST99 produced by *L. mesenteroides* subsp. *dextranicum* ST99 (Todorov *et al*., 2004), the African fermented foods B.14 described for *L. lactis* subsp. *lactis* B.14 (Von Mollendorff *et al*., 2006) and *Leuconostoc lactis* T196 obtained from African *Colocynthis citrullus* (Bello *et al*., 2016)

It was found that in the combined application of the sublethal levels of clinical antibiotic (rifampicin) and the bacteriocin, antibacterial activity was strongly increased. The antibacterial activity exerted by bacteriocin DZ2 at concentration of 160 AU ml^{-1} was similar to that of rifampicin at the concentration of 0.2 µg ml^{-1} and showed no significant difference from the control ($P > 0.05$). There exist a good synergistic relationship between the bacteriocin and rifampicin at all concentrations as stronger antibacterial effect was mounted against *L. monocytogenes* NCTC 4885 when the two agents were combined.

The results obtained about the leakage of DNA, RNA, proteins and β-galactosidase confirmed that bacteriocin DZ2 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 and Dicks, 2006b; Todorov *et al*., 2007; Todorov and Dicks, 2005b).

Incubation of the producer cells in the presence of 100 mM NaCl at pH 2.0 did not result into detection of activity of bacteriocin DZ2, suggesting that these three 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 and Dicks, 2006a). In a similar study, 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 (Von Mollendorff *et al*., 2006).

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* which was insensitive.

This study showed that seeds of *I. gabonensis* contained bacteriocin-producing LAB that can be used as bio-preservatives. It suggested that bacteriocins serve as alternatives to classical antibiotics in treating bacterial infections. LAB naturally present in the fermented foods may contribute to the increase of the microbiological safety of the products. Bacteriocins produced by these LAB possess the potential to control food spoilage bacteria. The combined application of bacteriocins and rifampicin with synergetic activity may be an answer in better control of the human and animal pathogens. The study suggested an alternative means that could be explored to reduce the level of the minimal inhibitory concentrations (MIC) of the rifampicins (which could be applied to other antibiotics) when combined with natural peptides produced by lactic acid bacteria. This synergetic effect possesses the potential to reduce destruction of cells, toxicity and some other side effects that are usually associated with the consumption of antibiotics, especially in large doses. So, in the future, combination of antibiotics with antimicrobial peptides could allow for reduced use of antibiotics in medical applications and could help to prevent the emergence of bacteria resistant to antibiotics.

Acknowledgement

Tertiary Education Trust Fund (TETFund) was utilised for this research through Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria. I appreciate the Team Leader of Microbial Biotechnology Laboratory and the authority of Department of Biological Sciences, Faculty of Agriculture, Science and Technology, North West University, Mafikeng Campus, South Africa. Thanks to Todorov SD and others whose publications have been helpful.

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References

1 Ladipo DO Fondour JM, Garga N. Domestrication of bush mango (*Irvingia* sp). In International Centre for Research in Agro forestry. IRA/CRAF projects, 1995; 48.
2 Etebu E. Postharvest pathology and phytochemicals of *Irvingia gabonensis* (Aubry-Lecomte ex O'Rorke) fruits and wastes. *Journal of Agricultural Sciences Research*. 2012; 2(6):285-294.
3 Ngondi JL, Oben JE, Minka SR. The effect of *Irvingia gabonensis* seeds on body weight and blood lipids of obese subjects in Cameroon. *Lipids in health and Disease*, 2005; 4:12-15.
4 Bello OO, Bankole SA, Babalola OO. Bacteriocin detection from seeds of *Colocynthis citrullus* and evaluation of its antibacterial activities, 2016; 3(5): 215-248.
5 Oguntoyinbo FA, Narbad A. Molecular characterization of lactic acid bacteria and in situ amylase expression during traditional fermentation of cereal foods. *Food Microbiol*ogy, 2012; 31: 254-262.
6 Mayo B, Aleksandrzak-Piekarczyk T, Fernández M, Kowalczyk M, Álvarez-Martín P, Bardowski J. Updates in the metabolism of lactic acid bacteria. In F. Mozzi, R. R. Raya and G. M. Vignolo (Eds.), Biotechnology of lactic acid bacteria. Novel applications. Iowa, USA. Wiley-Blackwell, 2010; 3-33.
7 Macwana S, Muriana PM. Spontaneous bacteriocin resistance in *Listeria monocytogenes* as a susceptibility screen for identifying different mechanisms of resistance and modes of action by bacteriocins of lactic acid bacteria. *Journal of Microbiological Methods*, 2012; 88:7-13.
8 Gratia A. Sur un remarquable exemple d'antagonisme entre deux souches de coilbacille. *Comptes Rendus des Séances et Mémoires de la Société de Biologie*, 1925; 93:1040-1041.
9 Collins B, Cotter PD, Hill C, Ross RP. Applications of lactic acid bacteria-produced bacteriocins. In F. Mozzi, R. R. Raya and G. M. Vignolo (Eds.), Biotechnology of lactic acid bacteria. Novel applications. Iowa, USA. Wiley-Blackwell, 2010; 89-109.
10 Franz CMAP, Cho GS, Holzapfel WH, Gálvez A. Safety of lactic acid bacteria. In F. Mozzi, R. R. Raya and G. M. Vignolo (Eds.), Biotechnology of lactic acid bacteria. Iowa, USA. Wiley-Blackwell. Novel applications, 2010; 341-359.
11 Todorov SD, Dicks LMT. Characterization of bacteriocins produced by lactic acid bacteria isolated from spoiled black olives. *Journal of Basic Microbiology*, 2005a; 45:312–322.
12 Todorov SD. Diversity of bacteriocinogenzic lactic acid bacteria isolated from boza, a cereal-based fermented beverage from Bulgaria. *Food Control,* 2010; 21:1011-1021.
13 Stiles ME, Holzapfel WH. Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, 1997; 36:1–29.
14 Felske A, Rheims H, Wolterink A, Stackebrandt E, Akkermans ADL. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology*, 1997; 143:2983–2989.
15 Schägger H, Von Jagow G. Tricine–sodium dodecyl sulphate– polyacrylamide gel electrophoresis for the separation of protein in the range from 1 to 100 kDa. *Analysis of Biochemistry*, 1987; 166:368–379.
16 Van Reenen CA., Dicks LMT. Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus* pentosus. *Current Microbiology* 1996; 32;183–187.
17 Todorov SD, Dicks LMT. Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria. Comparison of the bacteriocins. *Process Biochemistry,* 2006a; 41:11–19.
18 Todorov SD, Danova ST, Vamn Reenen CA, Meincken M, Dinkova G, Ivanova IV *et al.* Characterization of bacteriocin HV219, produced by *Lactococcus lactis* subsp. *lactis* HV219 isolated from human vaginal secretions. *Journal of Basic Microbiology*, 2006a; 46:226–238.
19 Yang R, Johnson M., Ray B. A novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, 1992; 58:3355–3359.
20 Vaucher RA, Gewehr CCV, Correa APF, Sant'Anna V, Ferreira J, Brandelli A. Evaluation of the immunogenicity and in vivo toxicity of the antimicrobial peptide P34. *International Journal of Pharmaceutics*, 2011; 421:94-98.
21 De Vuyst L, Vandamme EJ. Bacteriocins of lactic acid bacteria: Microbiology, genetics and applications. London: Blackie Academic and Professional, 1994; 97-101.
22 Wachsman MB., Castilla V, de Ruiz Holgado AP, de Torres PA., Sesma F, Coto CE. Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro. *Antiviral Research*, 2003; 58:17–24.
23 Todorov SD, Wachsman MB, Knoetze H, Meincken M, Dicks LMT. An antibacterial and antiviral peptide produced by Enterococcus mundtii ST4V isolated from soy beans. *International Journal of Antimicrobial Agents*, 2005; 25:508–513.
24 Von Mollendorff JW, Todorov SD, Dicks LMT. Comparison of bacteriocins produced by lactic acid bacteria isolated from boza, a cereal-based fermented beverage from the Balkan Peninsula. *Current Microbiology*, 2006; 53:209–216.
25 Ivanova I, Kabadjova P, Pantev A, Danova S, Dousset X. Detection, purification and partial characterization of a novel bacteriocin substance produced by Lactoccous *lactis* subsp. *lactis* B.14 isolated from Boza – Bulgarian traditional cereal beverage. *Biocatalise*, 2000; 41:47–53.

26 Cheigh CI., Choi HJ, Park H, Kim SB, Kook MC, Kim TS, *et al.* Influence of growth conditions on the production of a nisin-like bacteriocin by *Lactococcus lactis* subsp. *lactis* A.264 isolated from kimchi. *Journal of Biotechnology*, 2002; 95:225–235.

27 Verellen TLJ, Bruggeman G, Van Reenen CA, Dicks LMT., Vandamme EJ. Fermentation optimisation of plantaricin 423, a bacteriocins produced by *Lactobacillus plantarum* 423. *Journal of Fermentation and Bioengineering*,1998; 86:174–179.

28 Todorov SD, Van Reenen CA, Dicks LMT. Optimization of bacteriocin production by *Lactobacillus plantarum* ST13BR, a strain isolated from barley beer. *Journal of General and Applied Microbiology*, 2004; 50:149–157.

29 Okkers DJ, Dicks LMT, Silvester M, Joubert JJ, Odendaal HJ. Characterization of pediocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus* pentosus with a fungistatic effect on Candida albicans. *Journal of Applied Microbiology*, 1999; 87:726–734.

30 Parente E, Ricciardi A. Production, recovery and purification of bacteriocins from lactic acid bacteria. *Journal of Applied Microbiology and Biotechnology*, 1999; 52:628-638.

31 Verschuere L, Rombaut G, Sorgeloos P, Verstraete W.. Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews,* 2000; 64(4):655-671.

32 Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. *Nature Reviews,* 2005; 3:777 - 788.

33 Drider D, Fimland G, Hechard Y, Mcmullen LM, Prevost H. The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews,* 2006; 70:564-582.

34 Gálvez A, Abriouel H, López RL, Ben Omar N. Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology*, 2007; 120(1-2):51-70.

35 Mojgani N, Amirinia C. Kinetics of growth and bacteriocin production in L. casei RN 78 isolated from a dairy sample in IR Iran. *International Journal of Dairy Science*, 2007; 2:1-12.

36 Bhunia AK, Johnson MC, Ray B, Kalchayanand N. Mode of action of pediocin AcH from Pediococcus acidilactici H on sensitive bacterial strains. *Journal of Applied Microbiology*, 1991; 70(1):25-33.

37 Todorov SD, Botes M, Danova ST, Dicks LMT. Probiotic properties of *Lactococcus lactis* subsp. *lactis* HV219, isolated from human vaginal secretions. *Journal of Applied Microbiology*, 2007; 103:629–639.

38 Todorov SD., Ho P, Vaz-Velho M, Dicks LMT. Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Science*, 2010; 84(3):334-343.

39 Belguesmia Y, Naghmouchi K, Chihib N. Class IIa bacteriocins: current knowledge and perspectives. In D. Drider, and S. Rebuffat (Eds.), Prokaryotic antimicrobial peptides. New York: Springer Publishing Company, 2011; 171-195.

40 Pinto AL, Fernandes M, Pinto C, Albano H. Castilho F, Teixeira P *et al*. Characterization of anti-Listeria bacteriocins isolated from shellfish: potential antimicrobials to control non-fermented seafood. *International Journal of Food Microbiology*, 2009; 129(1)50-58.

41 Keppler K, Geiser R, Holzapfel WH. An a-amylase sensitive bacteriocin of *Leuconostoc carnosum*. *Food Microbiol*ogy, 1994; 11:39–45.

42 Lewus CB., Sun S, Montville JT. Production of an a-amylase sensitive bacteriocin by an atypical Leuconostoc para*mesenteroides* strain. *Applied and Environmental Microbiology*, 1992; 58:143–149.

43 Parente E, Moles M, Ricciardi A. Leucocin F10, a bacteriocin from *Leuconostoc carnosum*. *International Journal of Food Microbiology,* 1996; 33:231–243.

44 Lee NK, Paik HD. Partial characterization of lacticin NK24, a newly identified bacteriocin of *Lactococcus lactis* NK24 isolated from Jeot-gal. *Food Microbiol*ogy, 2001; 18:17–24.

45 Todorov SD, Dicks LMT. Pediocin ST18, an anti-*Listeria*l bacteriocin produced by *Pediococcus pentosaceu*s ST18 isolated from boza, a traditional cereal beverage from Bulgaria. *Process Biochemistry*, 2005c; 40:365–370.

46 Gálvez A, Valdivia E, Abríouel H. Isolation and characterization of enterocins EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. *Archives of Microbiology*, 1998; 171:59–65.

47 Yildirim Z, Johnson MG, Winters DK. Purification and mode of action of bifidocin B produced by Bifidobacterium bifidum NCFB 1454. *Journal of Applied Microbiology,* 1999; 86:45–54.

48 Todorov SD. Dicks LMT. Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. *Biotechnology Journal*, 2006b; 1:405–409.

49 Todorov SD, Dicks LMT. Effect of growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a strain isolated from boza. *Food Technology and Biotechnology*, 2005b; 43:165–173.

50 Jillian EP. Bacteriocins and bacteriocin producers present in Kefir and Kefir grains; M.Sc Thesis, Department of Food Science, Stellenbosch University, 2006; 88-100.

51 Yildrim Z, Avsar YK, Yildrim M. Factors affecting the adsorption of buchnerin LB, a bacteriocin produced by *Lactobacillus bucneri*. *Microbiological Research,* 2002; 157:103-107.

6/5/2016