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Website: www.aarf.asia Email : editor@aarf.asia , editoraarf@gmail.com

BACTERIOCIN DETECTION FROM SEEDS OF *COLOCYNTHIS CITRULLUS* AND EVALUATION OF ITS ANTIBACTERIAL ACTIVITIES

Olorunjuwon O. Bello[a,b], Samuel A. Bankole[a] and Olubukola O. Babalola[b]

[a]Department of Microbiology, Faculty of Science, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria.

[b]Department of Biological Sciences, Microbial Biotechnology Laboratoty, Faculty of Agriculture, Science and Technology, North West University, Mafikeng Campus, South Africa.

ABSTRACT

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria. Several bacteriocins have been characterized biochemically and genetically. However, isolating bacteriocin producers from African protein-rich food such as Colocynthis citrullus, characterizing their bacteriocins based on their structural characteristics, synergetic effects determination with their chemotherapeutic potentials are yet to be explored. One hundred samples of the food were purchased from different markets in southwestern Nigeria. Samples were serially diluted and plated on appropriate culture media using standard techniques. Bacteriocin activities were measured by spectrophotometric analysis. Student t-test and Analyses of Variance were employed for data analysis. Isolated bacteria were Bacillus spp., Streptococcus spp., Staphylococcus aureus, Proteus mirabilis, Serratia spp, Escherichia coli, Leuconostoc lactis, Pediococcus pentosaceus, Leuconostoc mesenteroides and Leuconostoc pseudomesenteroides. Leuconostoc lactis T196 was the bacteriocigenic bacterial strain characterized in this study. This inhibited the growth of standard organisms. The synergistic effect of characterized bacteriocin and rifampicin on standard organisms showed significant differences ($P < 0.05$).This study showed that C. citrullus is a rich source of bacteriocin with increased antibacterial activity. This study

suggested that bacteriocins serve as alternatives to classical antibiotics in treating bacterial infections, and their application in food preservation is inevitable.

Keywords: Bacteriocin, protein-rich food, *C. citrullus*, bio-preservatives, anti-bacteria.

1.0 Introduction

Bacteriocins are generally recognized as "natural" compounds able to influence the safety and quality of foods. Nowadays, consumers are particularly aware of the health concerns regarding food additives; the health benefits of "natural" and "traditional" foods, processed with no added chemical preservatives, are becoming more and more attractive. Bacteriocins, although showing antibiotic properties, are not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics that can determine allergic reactions in humans (Cleveland, Montville, Nes and Chikindas, 2001). Since the discovery of the first bacteriocin by Gratia in 1925 (Garneau, Martin and Vederas, 2002), bacteriocin production has been found in numerous species of bacteria, among which, due to their "generally recognised as safe" (GRAS) status, LAB have attracted great interest in terms of food safety.

C. cintrullus seeds are the fat- and protein-rich seeds of certain cucurbitaceous plants. Melon most popularly called "*Egusi*" in Nigeria goes by various botanical names according to its variety; these include "*Citrulus edulus, Colocynthis citrullus, Citrulus vulgaris, Citrulus lanatus,* as reported by Odigboh (1977) and Okoko (1997). *C. lanatus* (*Egusi*) is indigenous to the West African region; although, it is the progenitor of the watermelon, it was domesticated only for its seeds in West Africa (Blench, 1997). Processing of melon involves depodding, fermentation, washing drying, cleaning and shelling (Kushwaha, Strivastava and Singh, 2006). Depodding and fermentation are carried out simultaneously as the pods are left on the field to rot for three to four days; after the pods are rotten and soft, the washing stage is then initiated.

One major problem that besets melon seeds is that it deteriorates quickly in storage due to fungal infection (Aboaba and Amasike, 1991; Bankole, 1993; Bankole, Lawal and Adebanjo, 2004). To reduce quality loss in stored products, rapid drying to low moisture is often emphasized, because all scenarios leading to mould contamination and subsequent damage relate to non-maintenance of stored products at safe moisture content (Awuah and Ellis, 2002; Bankole and Adebanjo, 2003; Bankole, Osho, Joda and Enikuomehin, 2005). This work aims at determining the bacteriocin production of bacteria in seeds of *Colocynthis*

citrullus with the characterization of putative bacteriocins and determination of spectra of activities.

2.0 Materials and Methods

2.1 Sources of Samples, Processing and Bacteriological Analyses

One hundred samples of protein-rich *Colocynthis citrullus* seeds were purchased over a six-month period 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 carried out within 24 h in the Microbiology Laboratory, Department of Microbiology, Olabisi Onabanjo University, Ago-Iwoye, Ogun State, Nigeria. Ten grams of ground sample were added to 90 ml of 0.1% (W/V) sterilized peptone water in a beaker and allowed to stand for 5 mins with occasional stirring. The samples were homogenized and appropriate dilutions of up to 10^{-8} were plated in duplicates on surfaces of respective media for microbial count using the spread plate technique (Ogunshe *et al*., 2006; Ogunshe and Olasugbo, 2008).

Aerobic mesophilic bacteria (AMB) were counted on plate count agar (PCA); violet red bile agar (VRBA) was employed for the enumeration of coliforms. Purplish red colonies surrounded by reddish zone of precipitated bile were counted as coliforms. Enterobacteriaceae were counted on MacConkey. Pink to red purple colonies with or without haloes of precipitation were counted as member of Enterobacteriaceae. *Streptococcus* spp were isolated on blood agar plates with characteristic colonies showing β-haemolysis confirmed as streptococci. *Bacillus* spp was isolated on mannitol/eggs yolk/polymyxin agar (MYP). MYP was prepared using peptone (Oxoid, U.K.), meat extract (Oxoid, U.K.), D-mannitol, sodium chloride, phenol red, agar-agar, egg yolk (Oxoid, U.K.) and polymyxin B sulphate (Pfizer). The plates were incubated at 32°C for 48 h (Barbier *et al.,* 2010). All catalase positive, motile organisms with ellipsoidal spores and positive V-P reaction were confirmed as *B. cereus*. Staphylococci were counted on mannitol salt agar (MSA) after incubation at 32^{0}C for 48 hours (Barbier *et al.*, 2010).

2.2 Characterization and identification of isolates

Representative colonies were randomly picked from countable plates of PCA and MacConkey agar plates, and further purified by repeated plating on PCA (Holt *et al.* 1994). Pure cultures were preserved on nutrient agar slants at the appropriate refrigeration temperature (4°C). Cell morphology, Gram's reaction, colony characterization and biochemical characterizations of isolates were performed according to standard procedures

(Cowan and Steel, 1985; Holt *et al.* 1994). API 20E and API 20NE were used for additional identification of members of Enterobacteriaceae and non-entrobactericeae, respectively.

2.3 Determination of pH

pH was measured using digital pH meter (Nig 333, Naina Solaris LTD, India) after homogenizing 10 grams of the protein-rich food product in 90 ml of peptone water.

2.4 Isolation of lactic acid bacteria and screening for bacteriocin-producing isolates

Samples of *C. citrullus* were screened for bacteriocinogenic lactic acid bacteria as described by Todorov and Dicks (2005a). 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).

2.5 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 (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* (Sneath *et al.*, 1986). 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′; Lee *et al.*, 2000). 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 94^0C, and 1 min at 36^0C, followed by an increase to 72 ^{0}C over 2 min. Extension of the amplified product was at 72^0C 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 37^0C, 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.).

2.6 Bacteriocin production

MRS broth (Biolab) was inoculated with a 24-h-old culture (2%, v/v) of a bacteriocin-producing strain of *Leuconostoc lactis* T196. Incubation was at 30^0C, 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 30^0C or 37^0C, respectively (Todorov, 2010).

2.6.1 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 80^0C. Ammonium sulfate was added slowly to the cell-free supernatant to 80% for *Leuconostoc lactis* T196, stirred for 4 h at 4^0C and then centrifuged (10,000g, 1 h, 4^0C). 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 (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 Van Reenen *et al.* (1998). *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.

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

Cell-free supernatant of bacteriocin-producing strain, obtained by centrifugation (8000g, 10 min, 4^0 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 30^0C, 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 30^0, 37^0, 45^0, 60^0 and 100^0C, respectively. Residual bacteriocin activity was tested after 30, 60 and 120 min at each of these temperatures, as described before (Todorov *et al.*, 2006; Todorov, 2010).

2.6.3 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 bacteriocin T196, for 5 min at 25^0C, 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 37^0C, 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, 25^0C) 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.*, 2006; Todorov, 2010).

2.6.4 Combined effect of bacteriocins, rifampicin and pH on growth of *L. monocytogens* NCTC 4885

L. monocytogens NCTC 4885 was grown in BHI broth for 18 h at 37^0C. All tests were conducted in sterile STERELIN™ micro titer plates. Each well was filled with 190 µl of BHI medium at pH to 4.0, 5.0, 6.0 and 7.0 (corrected with 1 M lactic acid). A combination of different concentrations of rifampicin (Sigma) under MIC (final concentration 0.2 μgml^{-1}; 0.1 μgml^{-1} and 0.05 μgml^{-1}) and bacteriocin (final concentration 160 AU ml^{-1}, 80 AU ml^{-1}, 40 AUml^{-1} and 16 AU ml^{-1}) were added to the micro titer wells. The wells were inoculated with 10 µl culture of *L. monocytogens* NCTC 4885 (OD_{600}nm = 0.3). Optical density readings (at 595 nm) were recorded every hour for 12 h on a Bio-Rad micro plate reader. Cultures grown in BHI broth, without added bacteriocins and rifampicin, served as a control (Gillor, Nigro and Riley, 2005; Todorov, 2010).

2.6.5 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 30^0C, 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 4^0C and then harvested (12,000g, 15 min, 4^0C). 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 *et al.*, 2011)

2.6.6 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 bacteriocins and antibiotic (rifampicin) against *L. monocytogens* NCTC 4885 using SPSS version 17.0.

3.0 Results

Figure 1 showed the percentage occurrence of Gram negative and Gram positive bacteria, including lactic acid bacteria, in *Colocynthis citrullus*. Among the three Gram negative isolates, *P. mirabilis* had the highest percentage frequency of 47.1%, followed by *E. coli* (35.2%) and *Serratia* spp (17.7%). In similar vein, three Gram positive isolates which were *Bacillus* spp, *Streptococcus* spp and *Staphylococcus aureus* had percentage occurrences of 49.3%, 19% and 31.7%, respectively. *Leuconostoc lactis*, a bacteriocigenic strain among LAB isolates, had percentage occurrence of 36.9% while *Pediococcus pentosaceus*, *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* had percentage frequencies of 18%, 19.8% and 15.3%, respectively.

Table 1 shows the spectrum of activities of *L. lactis* T196 against certain Gram-positive and Gram-negative bacterial strains. Out of 45 indicator bacterial strains investigated, 30 (66.67%) were sensitive to T196.

Figure 3 illustrates the growth of *L. lactis* T196 in association with bacteriocin production at varying cell densities. Bacteriocin level of activity was as high as 5,000 AU ml^{-1} just at the 6^{th} hour of fermentation and increased to 50,000 AU ml^{-1} at the 15^{th} hour, but decreased to 25,000 during the following 9 hours. Cell density measured to 5.0 OD_{600} nm.

Figure 4 shows the tricine-SDS–PAGE of bacteriocins T196. The size of bacteriocin T196, as revealed by tricine-SDS–PAGE, was 3.2kDa. Table 2 shows the factors affecting the antimicrobial activity of bacteriocins T196. Cell-free supernant of bacteriocin T196 was subjected to treatments with some enzymes, surfactants, EDTA and varying pH and temperature values. The antimicrobial activity of 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 strain T196 was treated with α-amylase and catalase. Bacteriocin remained active after incubation at pH 2.0–8.0. 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.

Figures 5 and 6 showed the effects of bacteriocins produced by A) *Lactobacillus plantarum* strain Z1116, B) *Enterococcus faecium* AU02, C) *Leuconostoc lactis* PKT0003, D) *Leuconostoc lactis* T196 and E) *Leuconostoc lactis* DZ2 on the growth of *E. faecium* HKLHS and *L. sakei* DSM 20017, respectively. The modes of activity of these bacteriocins were bactericidal, as determined against *E. faecium* HKLHS and *L. sakei* DSM 20017, respectively.

Figure 7 shows effect of bacteriocin T196 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885. The antibacterial effect exerted on *L. monocytogenes* NCTC 4885 by bacteriocin T196 is also well established. The profile of inhibition of growth of the test organism by bacteriocin T196 at concentration of 160 AU ml^{-1}, was not significantly different ($P > 0.05$) as compared with that of rifampicin at sub-lethal concentration of 0.2 µg ml^{-1}. A sharp antimicrobial effect was mounted against the test organism at the 10^{th} hour and no growth occurred for the following two hours. The antibacterial activity improved when the bacteriocin and rifampicin at their different concentrations were combined. The synergistic activity was well established and the difference from the control was significant ($P < 0.05$).

Figure 8 shows the effect of bacteriocin T196, rifampicin and pH on the growth of *L. monocytogenes* NCTC 4885 at 37^0C. Increase in the antibacterial activity of the bacteriocin, at all concentrations against the test organism was recorded at the low pH. The synergistic effects of bacteriocins and rifampicin at different concentrations were also well established.

Table 3 showed 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

T196. The results obtained about the leakage of DNA, RNA, proteins and β-galactosidase confirmed that bacteriocins T196 destabilized the permeability of the cell membrane.

Figure 9 showed the percentage adsorption of bacteriocins T196 to cells of *E. faecium* HKLHS and *L. sakei* DSM 20017. Bacteriocin T196 activity was observed after treatment with 100 mM NaCl at pH 2.0 which suggested that the bacteriocin adsorbed to the cell-surface of the producers cells.

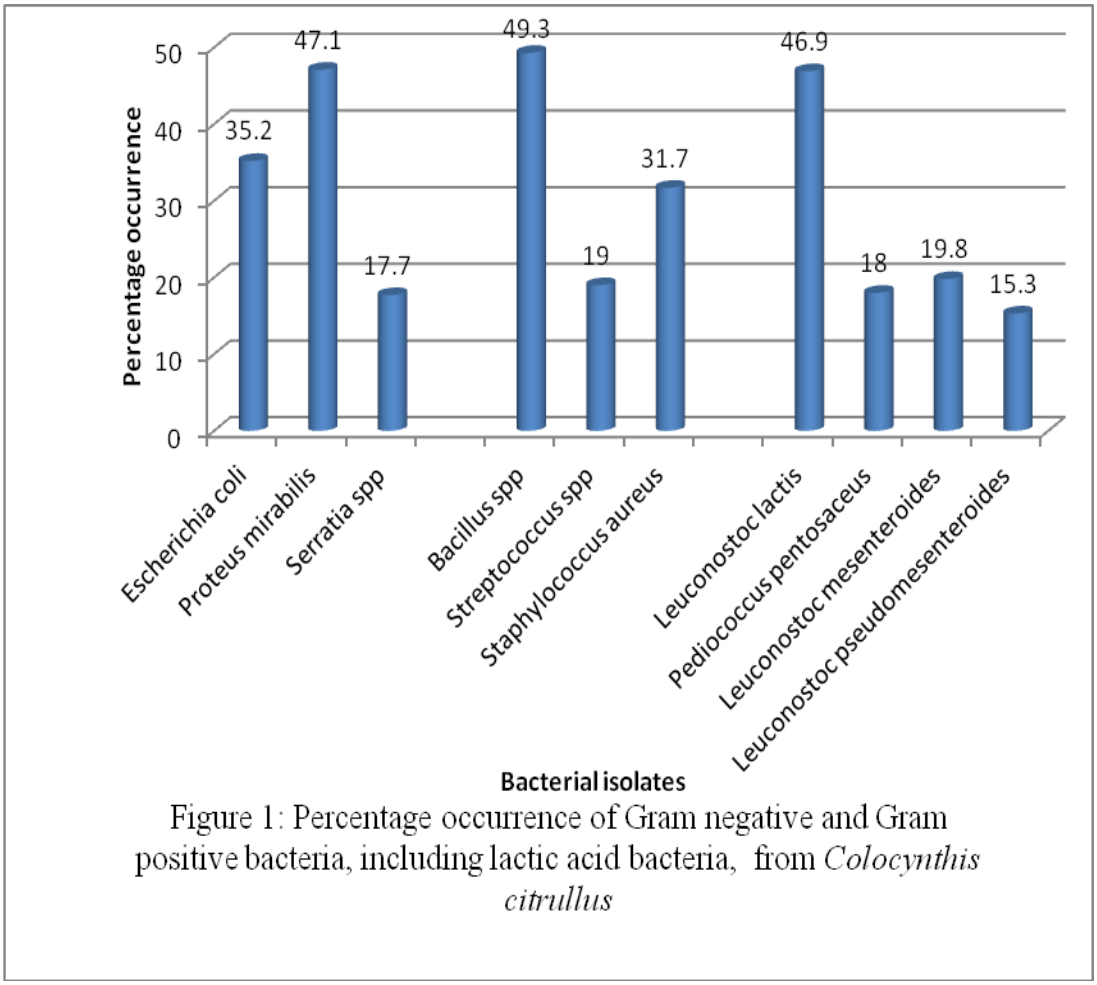

Figure 1: Percentage occurrence of Gram negative and Gram positive bacteria, including lactic acid bacteria, from *Colocynthis citrullus*

Table 1: Antibacterial spectrum of activity of bacteriocin produced by *L. lactis* T196 as examined with selected bacterial indicator strains

Bacterial Strain	Medium	Temperature (^{0}C)	*L. lactis* T196
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	-

Bacterial Strain	Medium	Temperature (^{0}C)	*L. lactis* T196
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.

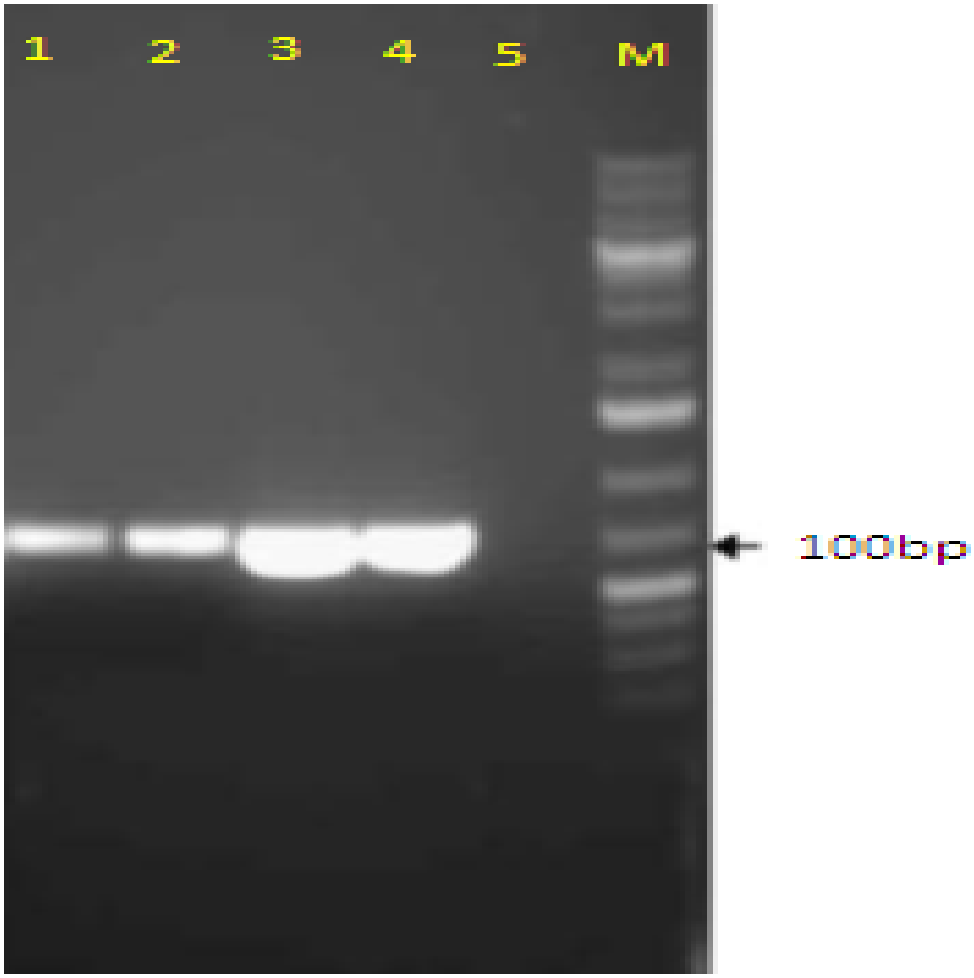

Figure 2: Agarose gels showing DNA fragments obtained after PCR with species-specific and genus-specific primers. Lanes 6 and 7: strain AU02, lane 8: *E. faecalis* PTA-7278 (ST4SA), lane 9: no DNA loaded, lane M2: O'GeneRuler™ Ultra Low Range DNA Ladder from Fermentas, Lanes 1 and 2: strain T196, lanes 3 and 4: *L. lactis* NCDO 533 (DSM 20202T), lane 5: no DNA loaded and lane M3: O'GeneRuler™ 1 kb DNA Ladder (Fermentas).

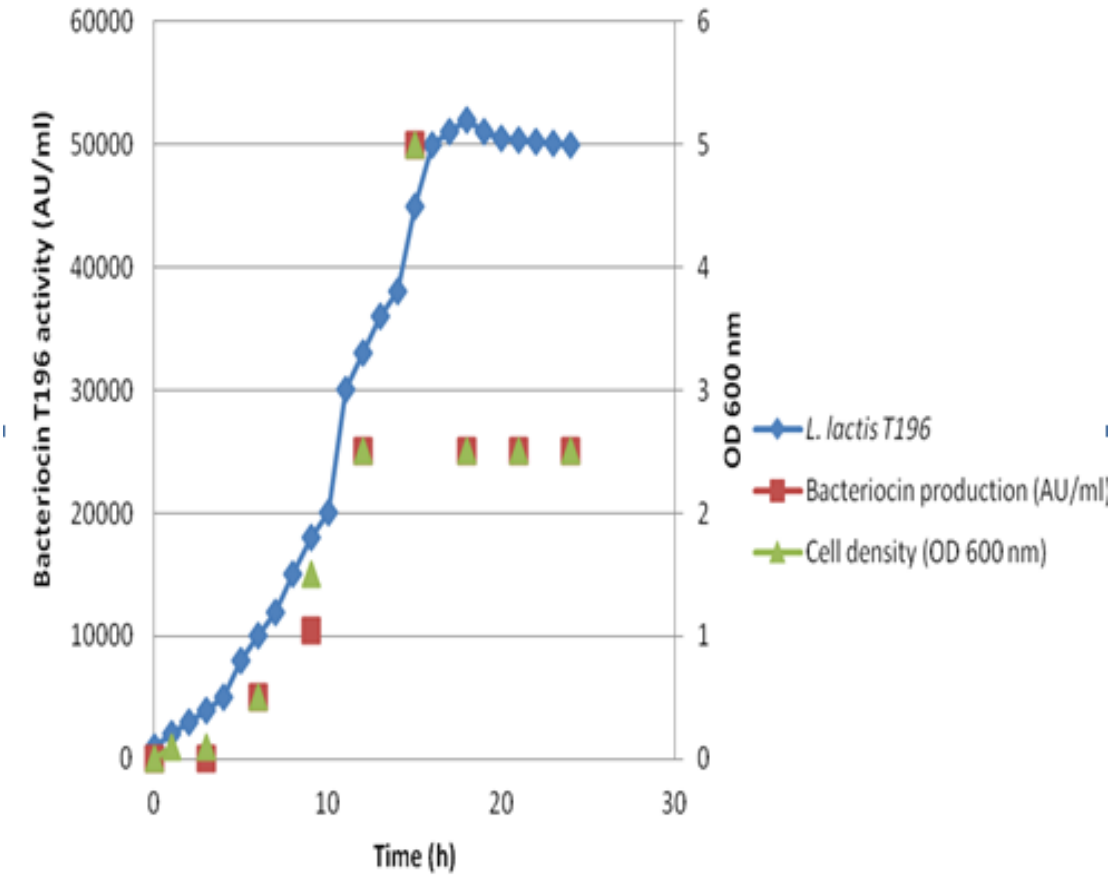

Figure 3: Growth of *Leuconostoc lactis* T196 in MRS broth in association with bacteriocin production at different cell densities

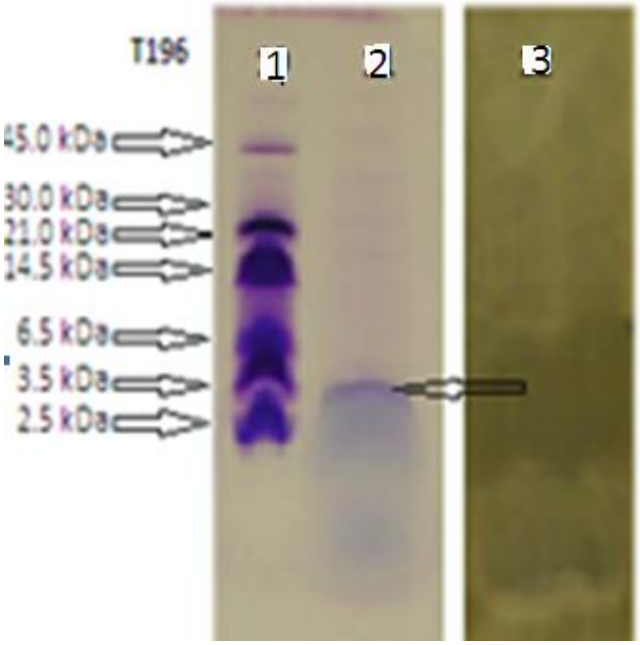

Figure 4: Tricine-SDS–PAGE of bacteriocins *L. lactis* T196. Lane 1: molecular mass marker (2.5–45.0 kDa, Amersham). Lanes 2: peptide band stained with Coomassie Blue R250. Lanes 3: zone of growth inhibition corresponding to the position of bacteriocin T196. The gel was overlaid with *E. faecium* HKLHS and *L. sakei* DSM 20017, respectively (ca. 1 x 10^6 CFU/ml) suspended in BHI and MRS agar.

Table 2: Factors affecting the antimicrobial activity of bacteriocin T196

Treatment	Bacteriocins
	T196
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

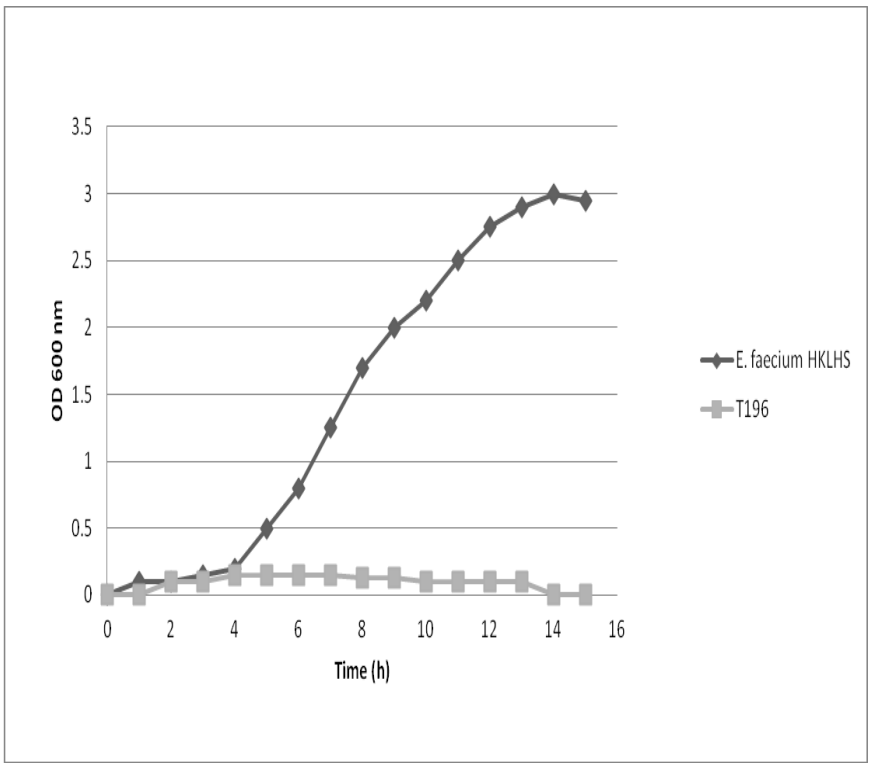

Figure 5: Effect of bacteriocin produced by *Leuconostoc lactis* T196 on the growth of *E. faecium* HKLS

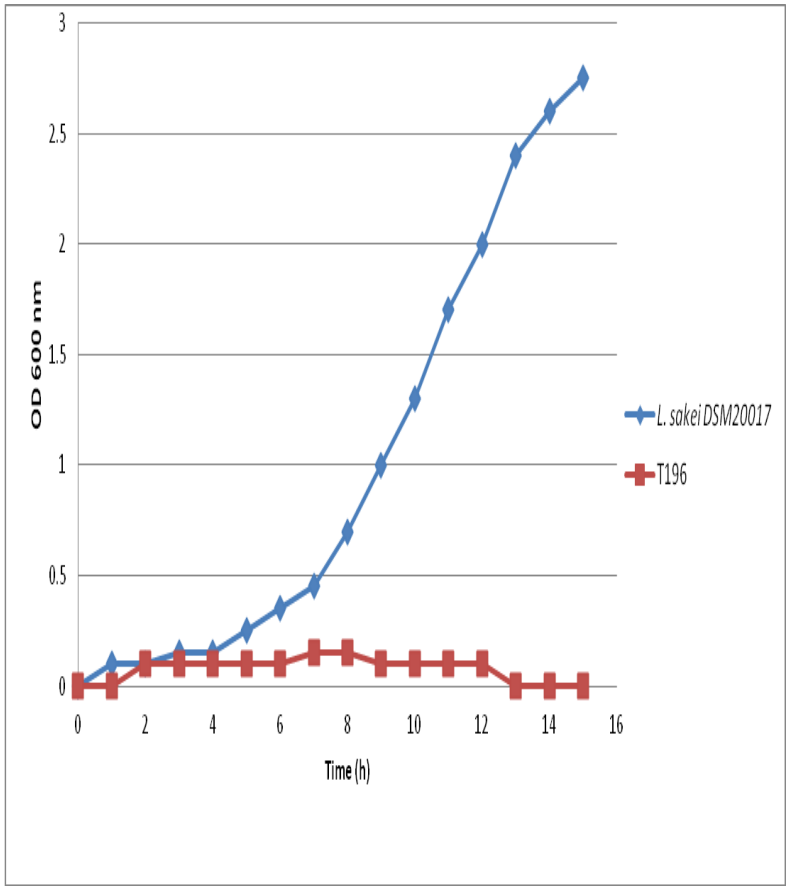

Figure 6: Effect of bacteriocin produced by *Leuconostoc lactis* T196 on the growth of *L. sakei* DSM20017.

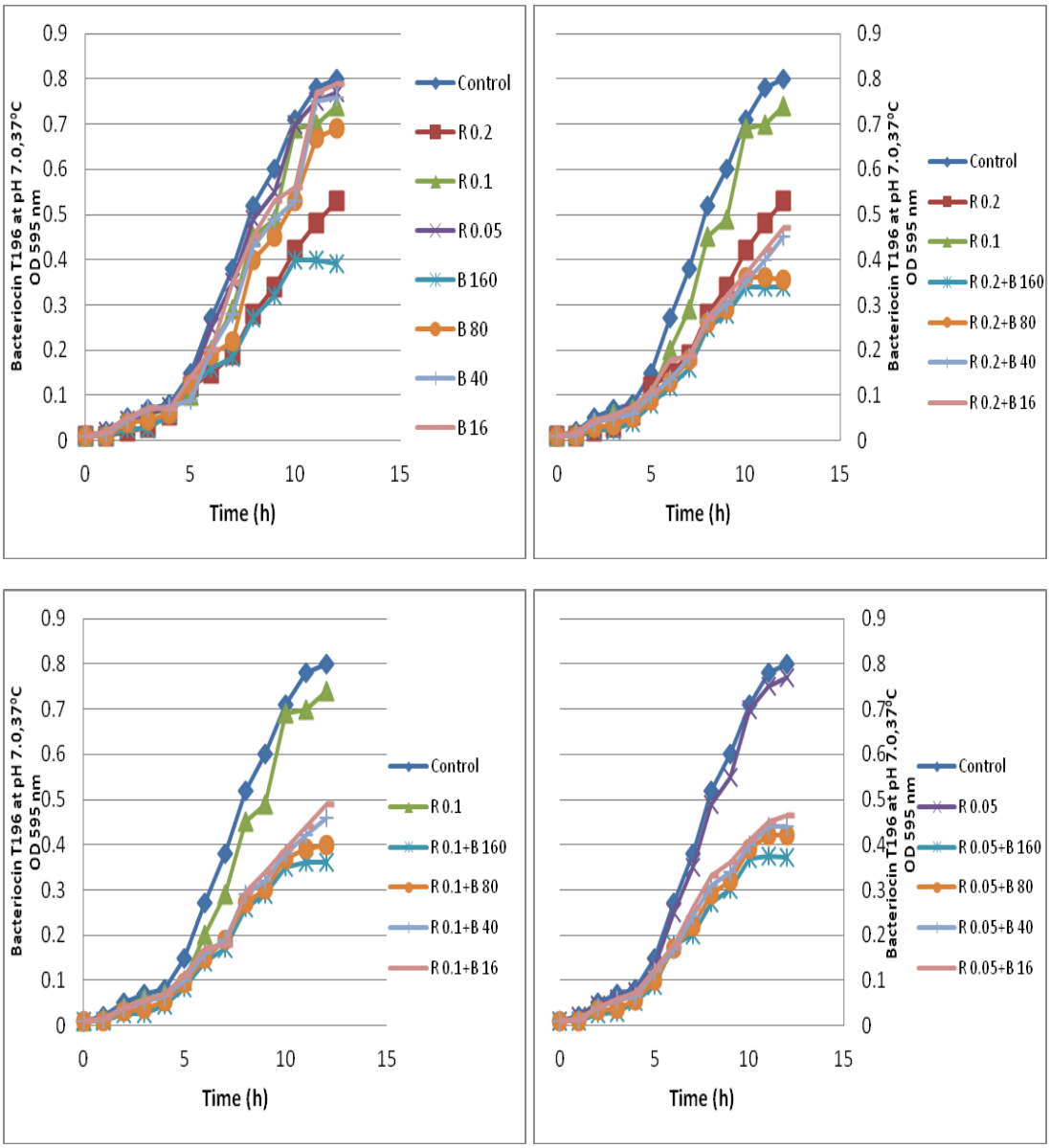

Figure 7: Effect of bacteriocin T196 and antibiotic (rifampicin) on the growth of *L. monocytogenes* NCTC 4885

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 T196 at concentration of 160 AU ml^{-1}; B 80=bacteriocin T196 at concentration of 80 AU ml^{-1}; B 40=bacteriocin T196 at concentration of 40 AU ml^{-1}; B 16=bacteriocin T196 at concentration 16 AU ml^{-1}

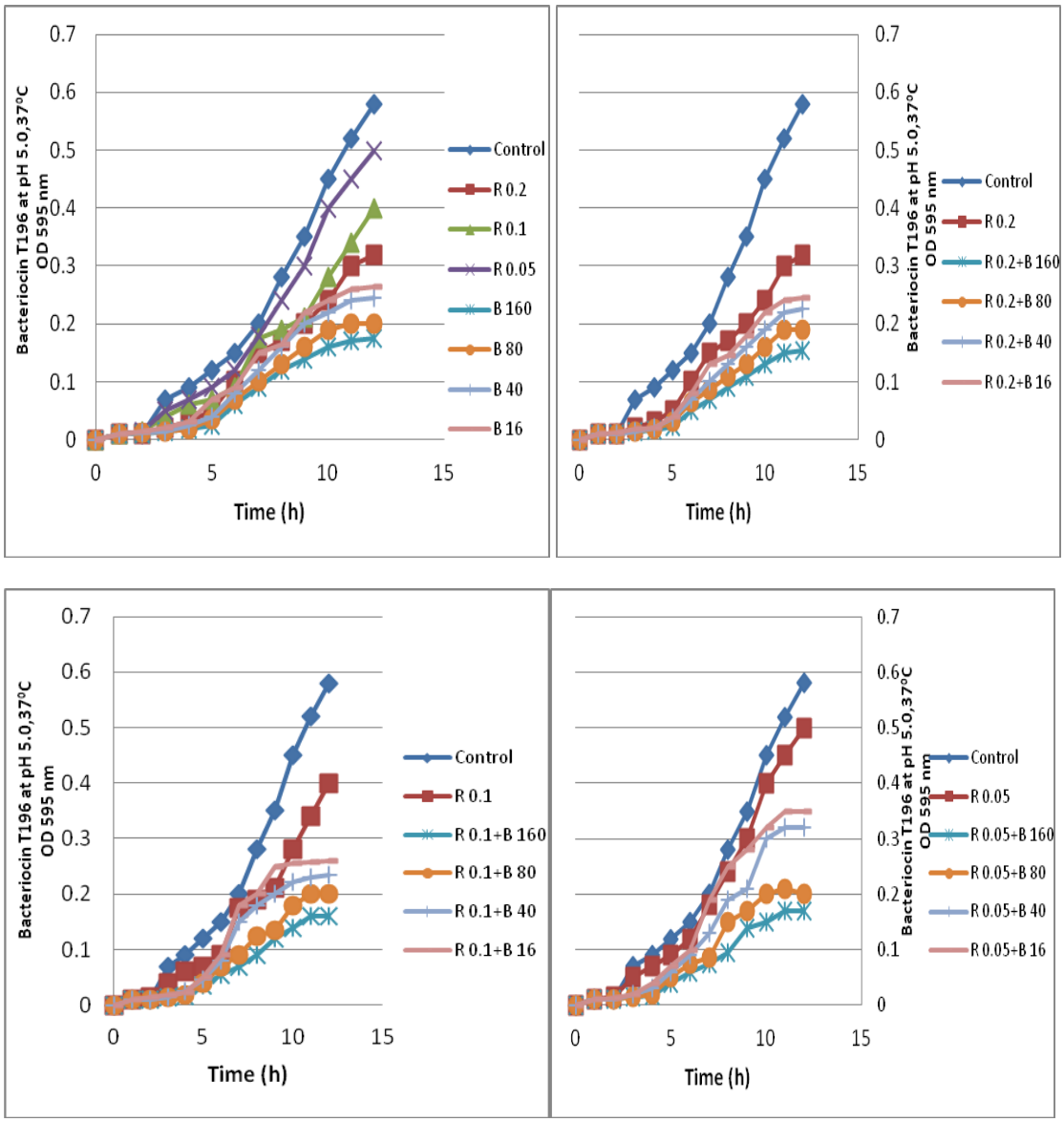

Figure 8: Effect of bacteriocin T196, rifampicin and pH on the growth of *L. monocytogenes* NCTC 4885

Control = no bacteriocin or rifampicin added to BHI with corrected to 5.0; 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 T196 at concentration of 160 AU ml^{-1}; B 80=bacteriocin T196 at concentration of 80 AU ml^{-1}; B 40=bacteriocin T196 at concentration of 40 AU ml^{-1}; B 16=bacteriocin T196 at concentration 16 AU ml^{-1}

Table 3: 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 T196

(A)	Intact cells of *E. faecium* HKLHS	Partially broken cells of *E. faecium* HKLHS	Bacteriocin	*E. faecium* HKLHS treated with bacteriocin
T196	0.023	0.172	0.085	0.105

(B)	Intact cells of *L. sakei* DSM 20017	Partially broken cells of *L. sakei* DSM 20017	Bacteriocin	*L. sakei* DSM 20017 treated with bacteriocin
T196	0.020	0.168	0.009	0.192

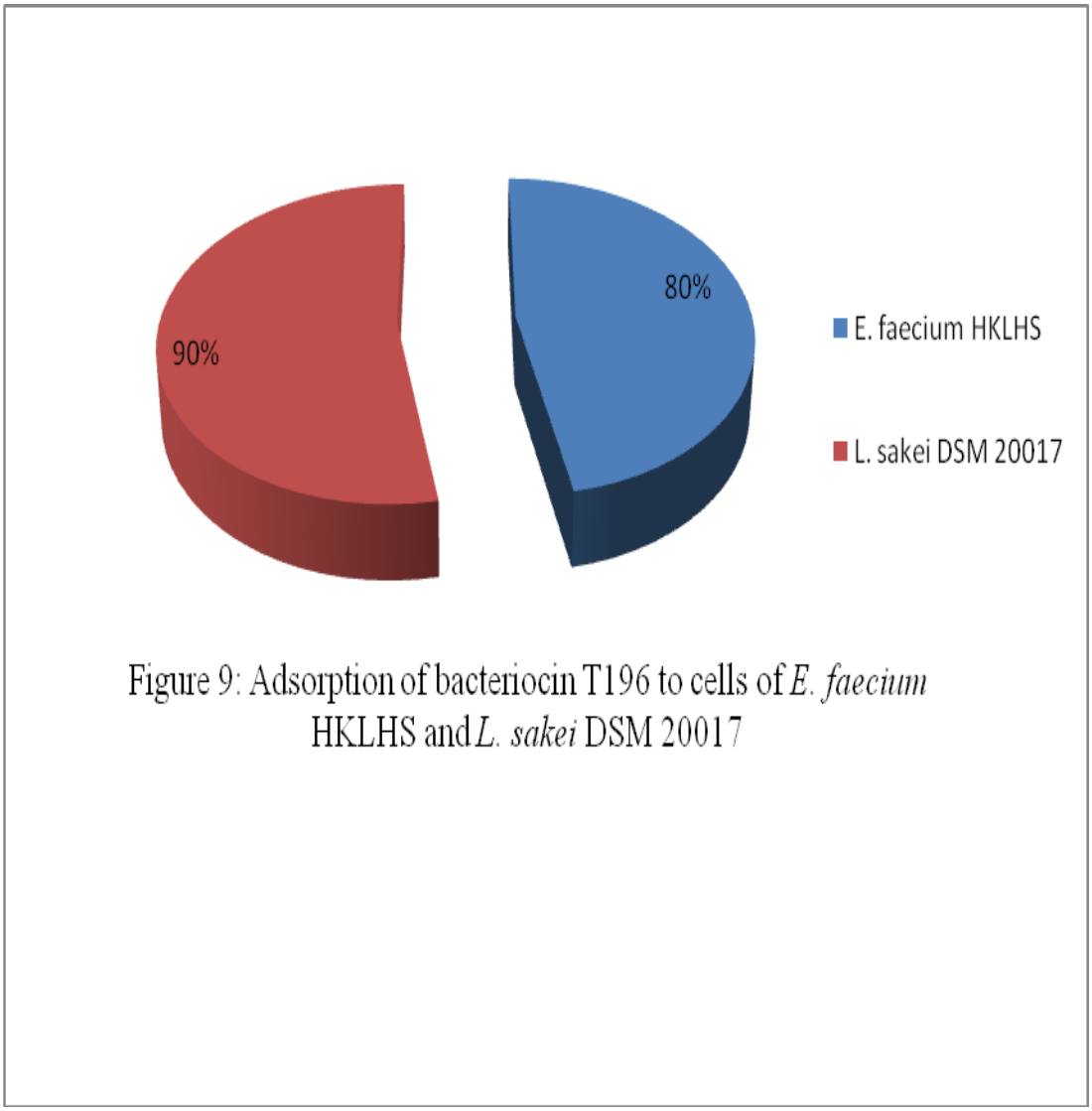

Figure 9: Adsorption of bacteriocin T196 to cells of *E. faecium* HKLHS and *L. sakei* DSM 20017

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The dynamics of fermentation in any food matrix is a complex microbiological process involving interactions between quite a number of different microorganisms, while the contribution of the accompanying flora of fermenting substrates is largely determined by the substrate composition and hygiene during production.

Six genera of bacteria isolated from *C. cintrullus* samples include *Streptococcus* spp, *Bacillus* spp, *S. aureus, Proteus mirabilis*, *Serratia* spp and *E. coli*. *Bacillus* species were reported by Owens *et al.* (1997) to be mostly responsible for the alkaline fermentation of condiments. The isolation of potential bacterial pathogens such as *E. coli, S. aureus* and *Salmonella* spp in this study could be associated with poor hygienic practices and poor qualities of water sources (Bello *et al.*, 2013a). The isolation and enumeration of *S. aureus* in food products is employed generally as a sanitation index (Bello *et al.*, 2013b).

The presence of these organisms in food beyond certain critical limit is interpreted as indicating that the food in question has been exposed to condition that might introduce or allow proliferation of pathogenic microorganisms (Mukhopadhyay, 2002). *S. aureus* is an important food-poisoning organism because of its cosmopolitan distribution in nature. Its presence in foods may be traced back to the environment as they are important normal flora of humans (Bergdoll, 1989; Newsome, 1998; Neihart *et al.*, 1998; Lin *et al.*, 2009).

S. aureus has been isolated from foods such as rice, spices, meat and dairy products (Sokari, 1991; Owhe-Ureghe *et al.*, 1993; Austin and Austin, 2007; Iwamoto *et al.*, 2010). With the exception of Ogundana (1980) who isolated fungal flora, and Bankole *et al.* (2005) who reported occurrence of *Aspergillus* spp in melon seeds, most workers have reported that only bacterial flora are associated with fermentations of protein seeds into food condiments.

Leuconostoc lactis T196 was a bacteriocigenic strain isolated from *Colocynthis citrullus* in this study. Other LAB isolates include *Pediococcus pentosaceus, Leuconostoc mesenteroides* and *L. pseudomesenteroides*. The bacteriocin 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 and Dicks, 2005a; Todorov, 2010) and viruses, e.g. herpes simplex virus and influenza (Wachsman *et al.*, 2003; Todorov *et al.*, 2005) 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 (von Mollendorff, Todorov and Dicks, 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, 2005b; Todorov and Dicks, 2006a). ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ were also reported (Todorov, 2010).

The activity of bacteriocins T196 was detected after 3 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; Todorov *et al.*, 2006). The decrease recorded in the production of bacteriocins T196 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. However, constant changes in the pH and medium composition during fermentation may lead to changes in activity levels of bacteriocins. The latter phenomenon has been reported for bacteriocins produced by *L. mesenteroides* L124, *L. curvatus* L442 (Mataragas *et al.*, 2003) and meat starter cultures (Nieto- Lozano *et al.*, 2002).

The size of bacteriocin T196 is 3.2 kDa. The size recorded for the bactericoin is within the range reported for most bacteriocins produced by *LactoBacillus* spp and *Enterococcus* spp (De Vuyst and Vandamme, 1994). This 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 properties of bacteriocin T196 found in this study allow their characterization as group IIa bacteriocin 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, Rombaut, Sorgeloos and Verstraete, 2000; Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Drider *et al.*, 2006; Gálvez, Abriouel, López and Ben Omar, 2007; Mojgani and Amirinia, 2007). 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 (Bhunia, Johnson, Ray, and Kalchayanand, 1991; Cotter *et al.*, 2005; Drider *et al.*, 2006; Todorov, Botes, Danova, and Dicks, 2007; Todorov, Ho, Vaz-Velho and Dicks, 2010; Belguesmia, Naghmouchi and Chihib, 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; Pinto *et al.*, 2009; Belguesmia *et al.*, 2011). This buttresses the broad spectra of activities exhibited by bacteriocin T196 characterized in this study.

The antimicrobial activity of bacteriocin T196 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 was treated with amylase and catalase. Bacteriocin T196 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 60^0C for 60 and 120 min.

Surfactants such as SDS, Tween 20, Tween 80, urea and Triton X-100 had no effect on the activity of bacteriocin T196. Addition of 1.0, 2.0 or 5.0 mM EDTA (final concentrations) did

not affect its activity. Complete inactivation of the bacteriocin was observed after treatment of the cell-free supernatants with proteolytic enzymes, confirming the proteinaceous nature of the antimicrobial compounds.

Treatment of cell-free supernatant with catalase and α-amylase did not result in activity changes, suggesting that the inhibition recorded was not hydrogen peroxide and that carbohydrate moieties were not required for antimicrobial activity. Stability of bacteriocin T196 in the presence of α-amylase is not unusual and similar results have also been reported for other bacteriocins (Todorov and Dicks, 2006a; von Mollendorff *et al.*, 2006). Leuconocin S (Keppler *et al.*, 1994) and carnocin 54 (Lewus, Sun and Montville, 1992) are sensitive to α-amylase, suggesting that their activity is associated with glycosylation of the active peptide. Thermostability at 100^0C has also been reported for most other bacteriocins (Todorov and Dicks, 2006a; von Mollendorff *et al.*, 2006; Todorov, 2010).

The mode of activity of bacteriocin T196 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 4 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 Dicks, 2005c), mesentericin ST99 produced by *L. mesenteroides* subsp. *dextranicum* ST99 (Todorov and Dicks, 2004), and the African fermented foods B.14 described for *L. lactis* subsp. *lactis* B.14 (Ivanova *et al.*, 2000).

The antibacterial effect exerted on *L. monocytogenes* NCTC 4885 by bacteriocin T196 was well established. The profile of inhibition of growth of the test organism by bacteriocin T196 at concentration of 160 AU ml^{-1}, was not significantly different ($P > 0.05$) as compared with that of rifampicin at sub-lethal concentration of 0.2 µg ml^{-1}. A sharp antimicrobial effect was mounted against the test organism at the 10^{th} hour and no growth occurred for the following two hours. The antibacterial activity improved when the bacteriocin and rifampicin at their different concentrations were combined. The synergistic activity was well established and the difference from the control was significant ($P < 0.05$).

When the experiment was conducted at a pH of BHI corrected to 5.0, a stronger effect between different concentrations of rifampicin and bacteriocins against the test microrganism was recorded. Results showed that low pH generally increased the antibacterial potency of the bacteriocin. Increase in the antibacterial activity of the bacteriocin, at all concentrations against the test organism was recorded at the low pH and this points to the idea that a combination between pH, antibiotics and bacteriocins is a possible approach for the control of growth of *L. monocytogenes* NCTC 4885.

In a similar study, Minahk, Dupuy and Morero (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, Friedrich, Zhang, Mendoza and Hancockm, 2002).

To the best of my knowledge, this is one of the first set of reports on the characterization of bacteriocin produced by LAB, and the synergetic effect between rifampicin and bacteriocin produced by *L. lactis* from African condiment, *Colocynthis citrullus*. Only the study of Minahk *et al.* (2004) was dedicated to the synergism between erythromycin, chloramphenicol, tetracycline and enterocin CRL35, produced by *E. faecalis* CRL35. The results obtained about the leakage of DNA, RNA, proteins and β-galactosidase confirm that bacteriocin T196 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 resulted into detection of activity of bacteriocin T196, which suggests that this bacteriocin adsorb to the cell-surface of the producers cells. 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.

5.2 CONCLUSIONS

This study showed that *C. cintrullus* is a rich source of bacteriocin-producing bacteria with antimicrobial activity against a number of food spoilage and pathogenic bacteria. LAB naturally present in the fermented foods may contribute to the increase of the microbiological safety of the products. This study suggested that bacteriocins serve as alternatives to classical antibiotics in treating bacterial infections, and their application in food preservation is inevitable. 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 also suggested an alternative means that could be explored to reduce the level of the minimal inhibitory concentrations (MIC) of 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 brought about by 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.

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References

Aboaba, O.O. and Amasike, J. (1991), Storage of melon seeds. *Nigerian Journal of Botany* 4, 213-219.

Austin, B. and Austin, D.A. (2007), Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, 4rd ed. Springer-Praxis, London, England. pp 34-46.

Awuah, R.T. and Ellis, W.O. (2002), Effects of some groundnut packaging methods and protection with *Ocimum* and *Syzygium* powders on kernel infection by fungi. *Mycopathologia* 154, 29-26.

Bankole, S.A. (1993), Moisture content, mould invasion and seed germinability of stored melon. *Mycopathologia* 122, 123-126.

Bankole, S.A. and Adebanjo, A. (2003), Mycotoxins in food in West Africa: Current situation and possibilities of controlling it. *African Journal of Biotechnology* 2(9), 254-263.

Bankole, S.A. Lawal, O.A and Adebanjo, A. (2004), Storage practises and aflatoxin B.1 contamination of '*Egusi*' melon seed in Nig. *Journal of Tropical Sciences* 44, 150–153.

Bankole, S.A. Osho, A. Joda, A.O and Enikuomehin, O.A. (2005), Effect of drying method on the quality and storability of '*Egusi*' melon seeds (*Colocynthis citrullus* L.) *African Journal of Biotechnology* 4(8), 799-803.

Barbier, F. Ruppé, E. Hernandez, D. Lebeaux, D. Francois, P. Felix, B. Desprez, A. Maiga, A. Woerther, P.L. Gaillard, K. Jeanrot, C. Wolff, M. Schrenzel, J. andremont, A. and Ruimy, R. (2010), Methicillin-resistant coagulase-negative staphylococci in the community: high homology of SCCmecIVa between *Staphylococcus epidermidis* and major clones of methicillin-resistant *Staphylococcus aureus*. *Journal of Infectious Diseases* 202, 270–281.

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

Bello, O.O., Bello, T.K. and Bankole, S.A. (2013b). Occurrence of antibiotic-resistant *Staphylococcus aureus* in some street-vended foods in Ogun State, Nigeria. *Journal of Advances in Biology,* **1**(1), 21-28

Bello, O.O., Osho, A., Bankole S.A .and Bello, T.K., (2013a), Bacteriological and Physicochemical Analyses of Borehole and Well Water Sources in Ijebu-Ode, Southwestern Nigeria. *IOSR Journal of Pharmacy and Biological Sciences,* **8** (2), 18-25.

Bergdoll, M.D. (1989). *Staphylococcus aureus*. In: Foodborne Bacterial Pathogens. Doyle M.P. (Ed). Marcel Dekker Inc. New York, pp. 463 – 524.

Bhunia, A.K., Johnson, M.C., Ray, B. and Kalchayanand, N. (1991), Mode of action of pediocin AcH from Pediococcus acidilactici H on sensitive bacterial strains. *Journal of Applied Microbiology*, 70(1), 25-33.

Carr, F.J., Chill, D. and Maida, N. (2002), The Lactic Acid Bacteria: A Literature survey. *Critical Review in Microbiology* 28, 281-370.

Cheigh, C.I., Choi, H.J., Park, H., Kim, S.B., Kook, M.C., Kim, T.S. *et al.* (2002). 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*, 95, 225–235.

Cleveland, J., Montville, T.J., Nes, I.F. and Chikindas, M.L. (2001), Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, 71, 1-20.

Collins, B., Cotter, P.D., Hill, C. and Ross, R.P. (2010), 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 pp. 89-109.

Cotter, P.D., Hill, C. and Ross, R.P. (2005), Bacteriocins: developing innate immunity for food. *Nature Reviews* **3**, 777 - 788.

Couce, A. and Blazquez, J. (2009), Side effects of antibiotics on genetic variability. FEMS *Microbiology*. Revew. 33, 531–538.

Cowan, S.T. and Steel, K.J. (1985), Manual for the identification of bacteria. Cambridge University Press, Verlage, New York, p. 502.

De Vuyst, L. and Vandamme, E.J. (1994), Bacteriocins of lactic acid bacteria: Microbiology, genetics and applications. London: Blackie Academic and Professional. pp. 97-101.

Drider, D., Fimland, G., Hechard, Y., Mcmullen, L.M. and Prevost, H. (2006), The continuing story of class IIa bacteriocins. *Microbiology and Molecular Biology Reviews*, 70, 564-582.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. and Akkermans, A.D.L. (1997), Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology*, 143, 2983–2989.

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

Garneau, S., Martin, N.I. and Vederas, J.C. (2002), Two-peptide bacteriocins produced by lactic acid bacteria. *Biochimie*, 84, 577-592.

Gillor, O., Nigro, L.M. and Riley, M.A. (2005), Genetically engineered bacteriocins and their potential as the next generation of antimicrobials. *Current pharmaceutical design*, 11, 1067-1075.

Ivanova, I., Kabadjova, P., Pantev, A., Danova, S. and Dousset, X. (2000), 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*, 41, 47–53.

Iwamoto, M., Ayers, T., Mahon, B.E. and Swerdlow, D.L. (2010), Epidemiology of seafood associated infections in the United States. *Clinical Microbiology Reviews* 23, 399–411.

Jillian, E.P. (2006), Bacteriocins and bacteriocin producers present in Kefir and Kefir grains; M.Sc Thesis, Department of Food Science, Stellenbosch University. Pp. 88-100.

Keppler, K., Geiser, R. and Holzapfel, W.H. (1994), An a-amylase sensitive bacteriocin of Leuconostoc carnosum. *Food Microbiol*ogy, 11, 39–45.

Kushwaha, H.L., Strivastava, A.P. and Singh, H. (2006), Development and Performance Evaluation of an okra seed Extractor. Agric. Eng. Int.: *Journal of Agricultural Engineering*, 5 (2), 9

Lewus, C. B., Sun, S. and Montville, J.T. (1992), Production of an a-amylase sensitive bacteriocin by an atypical Leuconostoc para*mesenteroides* strain. *Applied and Environmental Microbiology*, 58, 143–149.

Lin, J., Yeh, K.S., Liu, H.T. and Lin, J.H. (2009), *Staphylococcus aureus* isolated from pork and chicken carcasses in Taiwan: prevalence and antimicrobial susceptibility. *Journal of Food Protection* 72, 608-611.

Mataragas, M., Metaxopoulos, J., Galiotou, M. and Drosinos, E.H. (2003), Influence of pH and temperature on growth and bacteriocin production by Leuconostoc *mesenteroides* L124 and *Lactobacillus* curvatus L442. *Meat Science*, 64, 265–271.

Minahk, C.J., Dupuy, F. and Morero, R.D. (2004), Enhancement of antibiotic activity by sub-lethalconcentrations of enterocin CRL35. *Journal of Antimicrobials and Chemotherapy*, 53, 240–246.

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

Mukhopadhyay, A., Mitra, A., Roy, R. and Guha, A.K. (2002), An evaluation of street-vended sliced papaya (Carica papaya) for bacteria and indicator microorganisms of public health significance. *Food Microbiol*ogy, 19, 663-667

Neihart, R.E., Fried, J.S. and Hogdes, G.R. (1998), Coagulase-positive Staphylococci. *South Medical Journal*. 81, 491–500.

Newsome, R.I. (1998), *Staphylococcus aureus*. *Food Technology*, 42, 194 – 198.

Nieto-Lozano, J.C., Reguera-Useros, J.I., Pelaez-Martinez, M.C. and de la Torre, A.H. (2002), Bacteriocinogenic activity from starter culture used in Spanish meat industry. *Meat Science*, 62, 237–243.

Ogundana, S.K. (1980), The production of ogiri: Nigerian soup condiment. *Lebensmittel Wissenschaff und Technologia* 13, 33~336.

Ogunshe, A.A.O. and Olasugba, K.O. (2008), Microbial loads and incidence of food-borne indicator bacteria in most popular indigenous fermented food condiments from middle-belt and southwestern Nigeria. *African Journal of Microbiology Research* 2 (12), 332-339.

Ogunshe, A.A.O., Ayodele, A.I. and Okonko, I.O. (2006), Microbial studies on aisa, a potential indigenous laboratory fermented food condiment from Albizia saman (Jacq.) F. Mull. *Pakistan Journal of Nutrition*, **5** (1), 51-58.

Okkers, D.J., Dicks, LM.T., Silvester, M., Joubert, J.J. and Odendaal, H.J. (1999), Characterization of pediocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus* pentosus with a fungistatic effect on Candida albicans. *Journal of Applied Microbiology*, 87, 726–734.

Owens, J.D., Allagheny, N., Kipping, G. and Ames, J.M. (1997), Fermentation of volatile compounds during *Bacillus* subtilis fermentation of soya beans. *Journal of Science and Food Agriculture,* 74, 132-140.

Owhe-Ureghe, U.B., Ekundayo, A.O., Agbonlahor, D.E., Oboh, P.A. and Orhue, P. (1993), Bacteriological examination of some ready-to-eat foods marketed in Ekpoma, Edo State of Nigeria. *Nigerian Food Journal*. 11, 45-52.

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

Patrzykat, A., Friedrich, C.L., Zhang, L., Mendoza, V. and Hancockm, R.E.W. (2002), Sub-lethal concentrations of pleurocidin-derivedan timicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*, 46, 605–614.

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

Schägger, H. and Von Jagow, G. (1987), Tricine–sodium dodecyl sulphate– polyacrylamide gel electrophoresis for the separation of protein in the range from 1 to 100 kDa. *Analysis of Biochemistry*, 166, 368–379.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (1986), In P.H.A. Sneath and J.G. Jold (Eds.), *Bergey's manual of systematic bacteriology* Baltimore: Williams and Wilkins pp. 1071–1075.

Sokari, T. (1991), Distribution of enterotoxigenic *Staphylococcus aureus* in ready-to-eat foods in eastern Nigeria. *International Journal of Food Microbiology*, 12, 275-280.

Stiles, M.E. and Holzapfel, W.H. (1997). Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, 36, 1–29.

Todorov S.D. (2010), Diversity of bacteriocinogenic lactic acid bacteria isolated from boza, a cereal-based fermented beverage from Bulgaria. *Food Control* 21:1011-1021.

Todorov, S.D. and Dicks, L.M.T. (2004), Characterization of mesentericin ST99, a bacteriocin produced by Leuconostoc *mesenteroides* subsp. *dextranicum* ST99 isolated from boza. *Journal of Industrial Microbiology and Biotechnology*, 31, 323–329.

Todorov, S.D. and Dicks, L.M.T. (2005a), Characterization of bacteriocins produced by lactic acid bacteria isolated from spoiled black olives. *Journal of Basic Microbiology*, 45, 312–322.

Todorov, S.D. and Dicks, L.M.T. (2005b), Effect of growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a strain isolated from boza. *Food Technology and Biotechnology*, 43, 165–173.

Todorov, S.D. and Dicks, L.M.T. (2005c), Pediocin ST18, an anti-*Listeria*l bacteriocin produced by *Pediococcus pentosaceu*s ST18 isolated from boza, a traditional cereal beverage from Bulgaria. *Process Biochemistry*, 40, 365–370.

Todorov, S.D. and Dicks, L.M.T. (2006a), Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria. Comparison of the bacteriocins. *Process Biochemistry*, 41, 11–19.

Todorov, S.D. and Dicks, L.M.T. (2006b), Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. *Biotechnology Journal*, 1, 405–409.

Todorov, S.D., Botes, M., Danova, S.T. and Dicks, L.M.T. (2007), Probiotic properties of *Lactococcus lactis* subsp. *lactis* HV219, isolated from human vaginal secretions. *Journal of Applied Microbiology*, 103, 629–639.

Todorov, S.D., Danova, S.T., Van Reenen, C.A., Meincken, M., Dinkova, G., Ivanova, I.V. *et al.*, (2006), Characterization of bacteriocin HV219, produced by *Lactococcus lactis* subsp. *lactis* HV219 isolated from human vaginal secretions. *Journal of Basic Microbiology*, 46, 226–238.

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

Todorov, S.D., Van Reenen, C.A. and Dicks, L.M.T. (2004), Optimization of bacteriocin production by *Lactobacillus plantarum* ST13BR, a strain isolated from barley beer. *Journal of General and Applied Microbiology*, 50, 149–157.

Todorov, S.D., Wachsman, M.B., Knoetze, H., Meincken, M. and Dicks, L.M.T. (2005), An antibacterial and antiviral peptide produced by Enterococcus mundtii ST4V isolated from soy beans. *International Journal of Antimicrobial Agents*, **25**, 508–513.

Van Reenen, C.A. and Dicks, L.M.T. (1996), Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus* pentosus. *Current Microbiology*, 32, 183–187.

Vaucher, R.A., Gewehr, C.C.V., Correa, A.P.F., Sant'Anna, V., Ferreira, J. and Brandelli, A. (2011), Evaluation of the immunogenicity and in vivo toxicity of the antimicrobial peptide P34. *International Journal of Pharmaceutics*, 421, 94-98.

Verellen, T.L.J., Bruggeman, G., Van Reenen, C.A., Dicks, L.M.T. and Vandamme, E.J. (1998), Fermentation optimisation of plantaricin 423, a bacteriocins produced by *Lactobacillus plantarum* 423. *Journal of Fermentation and Bioengineering*, 86, 174–179.

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. (2000), Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews*,

Von Mollendorff, J.W., Todorov, S.D. and Dicks, L.M.T. (2006), Comparison of bacteriocins produced by lactic acid bacteria isolated from boza, a cereal-based fermented beverage from the Balkan Peninsula. *Current Microbiology*, 53, 209–216.

Yildirim, Z., Johnson, M.G. and Winters, D. K. (1999), Purification and mode of action of bifidocin B produced by Bifidobacterium bifidum NCFB 1454. *Journal of Applied Microbiology*, 86, 45–54.

Yildrim, Z., Avsar, Y.K. and Yildrim, M. (2002), Factors affecting the adsorption of buchnerin LB, a bacteriocin produced by *Lactobacillus bucneri*. *Microbiological Research* 157, 103-107.