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Full Length Research Paper

Antagonistic effect of brevicin on Gram positive and Gram negative food borne bacteria and its biopreservative efficacy in milk

Senbagam D.[1]*, Gurusamy R.[1] and Senthilkumar B.[2]

[1]Department of Biotechnology, Vivekanandha College of Engineering for Women, Tiruchengode-637 205, Tamil Nadu, India.
[2]Department of Biotechnology, Muthayammal College of Arts and Science, Rasipuram-637 408, Tamil Nadu, India.

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A new low molecular weight brevicin produced by *Lactobacillus brevis* NS01 has greater antimicrobial activity on Gram positive and negative food borne bacteria. This is stable at high temperature acidic to neutral pH, non proteolytic enzymes and organic solvents. The synergistic effect of brevicin with ethylenediaminetetraacetic acid (EDTA), sodium lactate (NaL) and potassium sorbate (KS) showed that better effect is with EDTA. Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) studies showed that the presence of aliphatic C-H stretching of fatty acids and this peptide has vinyl protons and β substituted aliphatic system. Their biopreservative efficacy was also proved using purified brevicin in milk and could control the bacterial growth in milk up to 18 days.

Key words: Antimicrobial activity, biopreservation, brevicin, Gram negative, Gram positive bacteria, lactic acid bacteria.

INTRODUCTION

The empirical use of microorganisms and their natural products for the preservation of foods (biopreservation) have been a common practice in the history of mankind (Galvez et al., 2007). Bacterial antagonism is of vital importance for the application of probiotic lactic acid bacteria (LAB). LAB that produce antimicrobial substances like lactic acid, hydrogen peroxide, diacetyl and small heat stable inhibitory peptides (bacteriocin) have considerable advantage in the competition with other microorganisms including pathogens (Venema et al., 1993; Hammami et al., 2010). Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or proteins, which have bactericidal or bacteriostatic effect on closely related species (Klaenhammer, 1993). Few bacteriocins have been found to be active against unrelated genera like *Clostridia*, *Listeria*, enteropathogenic bacteria and Gram negative bacteria, and those bacteriocins are promising candidates for biopreservation of food (Bredholt et al., 2001; Cleveland et al., 2001). Bacetriocins of LAB are considered as naturally safe preservatives, since it is assumed that they are degraded by proteases in gastrointestinal tract (Cleaveland et al., 2001; Cotter et al., 2005).

Food processors run the risk of significant economic losses due to food spoilage resulting from microbial contamination. In industrialized countries, the percentage of the population suffering from food borne disease each year has been reported to be up to 30% (WHO, 2007).

Although chemical preservatives may provide a solution,

*Corresponding author. E-mail: nevithasenthil@gmail.com. Tel: +91-94432 86292. Fax: +91-4288 234894.

Abbreviations: CEP, LAB, KS, NaL.

the use of such preservatives (nitrite) leads to negative consequences for human health (Mills et al., 2011). Bacteriocin in food preservation can offer several benefits like an extended shelf life of foods, decreased risk for transmission of food borne pathogens through the food chain, ameliorate the economic losses due to food spoilage and reduce the use of chemical preservatives (Thomas et al., 2000).

The possibility of incorporating the antimicrobial compound obtained from LAB directly into the food systems and pharmaceutical products (Stoyanova et al., 2006) and the approaches commonly used in the application of bacteriocins as biopreservative for food stuffs are inoculation of food with LAB that produce bacteriocin (Ennahar et al., 1999; Leory and Devuyst, 2006), addition of purified or semi purified bacteriocins as food preservative (Hartman et al., 2011) and the use of the product that is previously fermented with a bacteriocin producing strain (Susanne et al.,1986). In food systems, where the addition of living bacteria would be detrimental, cell free bacteriocin preparations like Nisaplin R (nisin A) or ALTA 23419 (pediocin PA-1) have been used. To date, nisin A is the only bacteriocin approved for commercial use in many countries. It clearly shows activity against wide spectrum of Gram positive and has a broad range of applications, though, due to its biochemical properties; it is best suited for use in acidic foods like processed cheese, soups or vegetables (Delvis, 2005).

The effect of nisin produced by LAB *in situ* against Gram positive microorganisms like *Listeria, Staphylococcus* and *Clostridium* was demonstrated and there are only few reports on Gram negatives. The effect of bacteriocin produced *in situ* against Gram negatives is of greater importance since most of the food borne pathogens are Gram negatives (Zottola et al., 1994). Therefore, research in characterization and application of bacteriocin as preservative has rapidly increased nowadays. This will pave the way for studying about many bacteriocins as biopreservative. In this report, new brevicin producing *L. brevis* NS01 isolated from goat milk has been described. The brevicin with antimicrobial activity against Gram negatives and positives were characterized, predicted their chemical structure and used as a biopreservative for milk to retard the bacterial spoilage.

MATERIALS AND METHODS

Cultivation and screening of bacteriocin producing bacteria

Goat milk sample was collected aseptically and 0.1 ml portion of respective dilution of homogenates (0.5% peptone solution) were spread on the MRS agar (De Man-Rogosa Sharpe, Hi media, India) plates and then incubated at 37°C for 48 h. Based on the colony morphology, pure culture of each isolate was made. Overnight culture of each isolate was inoculated into 25 ml of MRS broth and incubated for 24 h. Then, the broth culture was centrifuged at 8000 rpm for 10 min to collect the cell free supernatant. It was used to check the antimicrobial activity against *Bacillus coagulans* MTCC 1272 as indicator strain (Mallidis et al., 1990).

Taxonomic analysis of bacteriocin producing isolate

The selected isolates were identified by their morphological and biochemical characteristics. The isolate showed maximum antimicrobial activity was identified by 16S rRNA gene sequencing and phylogenetic analysis. The genomic DNA was isolated according to the method of Marmur (1961) and the 16S rRNA gene sequence was amplified using the primers (forward sequence: AGAGTTTGATCMTGGCTCAG and reverse sequence: GGTTACCTTGTTACGACTT). Purification of amplified 16S rRNA gene products and gel elution was done according to the procedure of Eppendorf perfect prep gel cleanup kit. The amplified 16S rRNA gene was sequenced as well as subjected to BLAST search tool for the closest match in the database. Phylogenetic analysis was performed by subjecting the deduced sequence to the 16S rRNA gene data base to obtain the closely related sequences, and the phylogenetic tree was constructed, based on evolutionary distances that were calculated by following the distance matrix method, using the Phylip package.

Preparation and analysis of crude extract of protein (CEP) for their antagonistic activity against food borne pathogens

24 h old MRS broth culture was prepared and the cells were collected by centrifugation (9,000 rpm for 15 min at 4°C) and washed twice in sodium phosphate buffer (pH 6). The pellets were then resuspended in 0.02 N HCl (pH 2) and heated at 100°C for 10 min. It was then cooled by keeping in ice and centrifuged at 15,000 rpm for 15 min. The supernatant was collected and its pH was adjusted to 6.0 to prevent the effect of organic acids (Todorov and Dicks, 2005) and it was filter sterilized by cellulose nitrate membrane (0.45 µm, Sartorius AG, Germany) to use as a CEP (Simova et al., 2009). The different concentrations of CEP (50 and 100 µl) were analyzed for their antimicrobial activity by well diffusion assay (Tukel et al., 2007) using food borne pathogens; *L. monocytogenes* MTCC 657, *B. coagulans* MTCC 1272, *S. aureus* MTCC 737, *Shigella flexeneri* MTCC 1457, *E. coli* MTCC 1687, *Proteus mirabilis* MTCC 425, *Salmonella typhi* MTCC 531, *Klebsiella pneumoniae* MTCC 530 and *Pseudomonas aeruginosa* MTCC 1688. All these strains were collected from Microbial Type Culture Collection (MTCC) and Gene Bank of Institute of Microbial Technology (IMTECH), Chandigarh, India. The antagonistic activity of these isolates was evaluated by measuring the resulting diameters of zone of inhibition in millimeters.

Purification and lyophilization of CEP

The CEP was first subjected to ammonium sulfate precipitation. Ammonium sulfate was added to CEP until the concentration of 60% (w/v) saturation then the precipitate was settled overnight. This was centrifuged at 6500 rpm in a refrigerated centrifuge for 30 min to collect the precipitate. It was then dissolved in 10 ml of 0.1 M acetate buffer (pH 5.0) and dialyzed against 500 ml of 0.1 M acetate buffer (pH 5.0) at 4°C for overnight (Coventry et al., 1996). The crude protein was purified by gel filtration chromatography using a cationic resin Sephadex G 100 (Hi media, India) (Akcelik et al., 2005). The crude protein was loaded onto a column (1.0 × 15 cm)

packed with Sephadex G 100 equilibrated with 0.1 M sodium acetate buffer (pH 5.0). After washing with buffer (2 times), the elution was performed with a negative linear gradient of 0 to 1.0 M NaCl (Aslam et al., 2011). The eluted compound was freeze dried using lyophilizer (Merk, India) for further study.

Bacteriocin assay

The titer was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of the indicator lawn. Thus, the arbitrary unit (AU) of antimicrobial activity/ml was defined as $2^n \times 1{,}000$ µl/10 µl (Yamamoto et al., 2003). To examine the activity units of brevicin, the crude extract as well as the purified form of protein was serially diluted two-fold with 0.1 M acetic acid. 50 µl from each dilution was added into the plates containing a lawn of *B. coagulans* and *S. typhi.*

Protein concentration determination

Protein concentration of crude protein and purified protein was determined by Bradford (1976) method using bovine serum albumin as the standard (Venema et al., 1993).

Characterization of CEP

To evaluate the thermal stability of the brevicin, the lyophilized protein sample (1600 µg ml^{-1}) was dissolved in acetate buffer (pH 6.0) and was heated at different temperatures ranging from 40 to 100°C (at increment of 10 units) for 30 min. The antimicrobial activity was checked at each of these temperatures by well diffusion agar method using *B. coagulans* and *S. typhi*. Likewise, the influence of pH was evaluated by adjusting the protein sample from 2 to 11 (at increments of 1 pH unit) with 1 M NaOH or 1 M HCl. Then, the antimicrobial activity was carefully checked (Bhattar-charya and Das, 2010). The effect of different enzymes like pro-tease, lipase diastase, pepsin, trypsin, catalase and amylase and organic solvents like ethanol and chloroform was determined by treating the solution with these enzymes and organic solvents (Tukel et al., 2007) separately. After 2 h, the antimicrobial activity was checked using *B. coagulans* and *S. typhi*. To determine the synergistic effect of brevicin with chelator like EDTA and other chemicals like sodium lactate (NaL) and potassium sorbate (KS), it was separately treated with EDTA (10 mM), NaL (2%) and KS (0.02%) (Ukuku and Fett, 2004). After 2 h, the antimicrobial activity was monitored by well diffusion method using Gram positives such as *S. aureus, L. monocytogens* and Gram negatives such as *E. coli, P. mirabilis* and *S. typhi.*

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli (1970) on a 5% polyacrylamide stacking gel and a 12% polyacrylamide-resolving gel. The protein marker (Medox Biotech, India) ranging from 3.0 to 6.6 kDa was used as a standard marker for the determination of molecular weight. Protein bands were stained by Coomasiee Brilliant Blue Staining.

FT-IR analysis

A Perkin-Elmer infrared spectrophotometer was used for the investigation of the surface functional groups. The samples with KBr (spectroscopic grade) pellets were prepared in the size of about 10 to 13 mm in diameter and 1 mm in thickness. The samples were scanned in the spectral range of 4000 to 400 cm^{-1}.

Proton nuclear magnetic resonance spectrum (^{1}H NMR) for the structural analysis of bacteriocin

The purified brevicin was lyophilized and dissolved in deuterated Dimethyl sulfoxide (DMSO). The proton NMR spectra were recorded, using the JEOL ECA 500 MHz spectrophotometer. The ^{1}H NMR measurements were carried out at the operating frequency of 400.134 MHz; spectral width, sw = 7246.381 Hz; acquisition time, at = 1.13 s; relaxation delay, d_1 = 1.0 s; T = 300 K and TMS as the internal standard.

Biopreservative efficiency of purified brevicin milk

About 100 ml of pasteurized (67°C for 20 min) milk was taken in 4 sets of sterile glass containers. Different concentrations like 1, 3, and 5% of brevicin were added to the first three bottles, respectively and the fourth one was kept as a control (without brevicin). Immediately, the microbial count was done by spread plate method and they were stored at refrigerated condition. Once in three days, the morphological and microbial load was observed for 18 days. The number of colony forming units were recorded and compared with the control (Vinodkumar et al., 2006).

Statistical analysis

The experiments were repeated thrice and the average data were submitted to analysis of variance using ANOVA (Minitab version 15). The significance of the differences was determined at ($p \leq 0.05$). The results were analyzed using sigma plot 10 (Systat Software Inc, USA).

RESULTS AND DISCUSSION

In this present work, goat milk sample was collected aseptically and a total of 10 different colonies were isolated. All were assessed for the production of bacteriocin on Muller Hinton Agar (Hi media, India) plates containing a lawn of *B. coagulans*. Among them, isolates 5, 7, 9 and 10 produced the zone of inhibition around *B. coagulans* were selected. *B. coagulans* is the microorganism most frequently associated to spoilage of canned vegetables with pH between 4 and 4.5 (Mallidis et al., 1990). Nineteen indicator strains including the genera of *Bacillus, Micrococcus, Listeria, Lactobacillus, Enterococcus, Leconostoc, E. coli, Pseudomonas, Salmonella, Clostridium* and *Pediococcus* was used to screen the bacteriocin producing organisms (Simova et al., 2009). Since the isolate 5 produced greater zone of inhibition than isolate

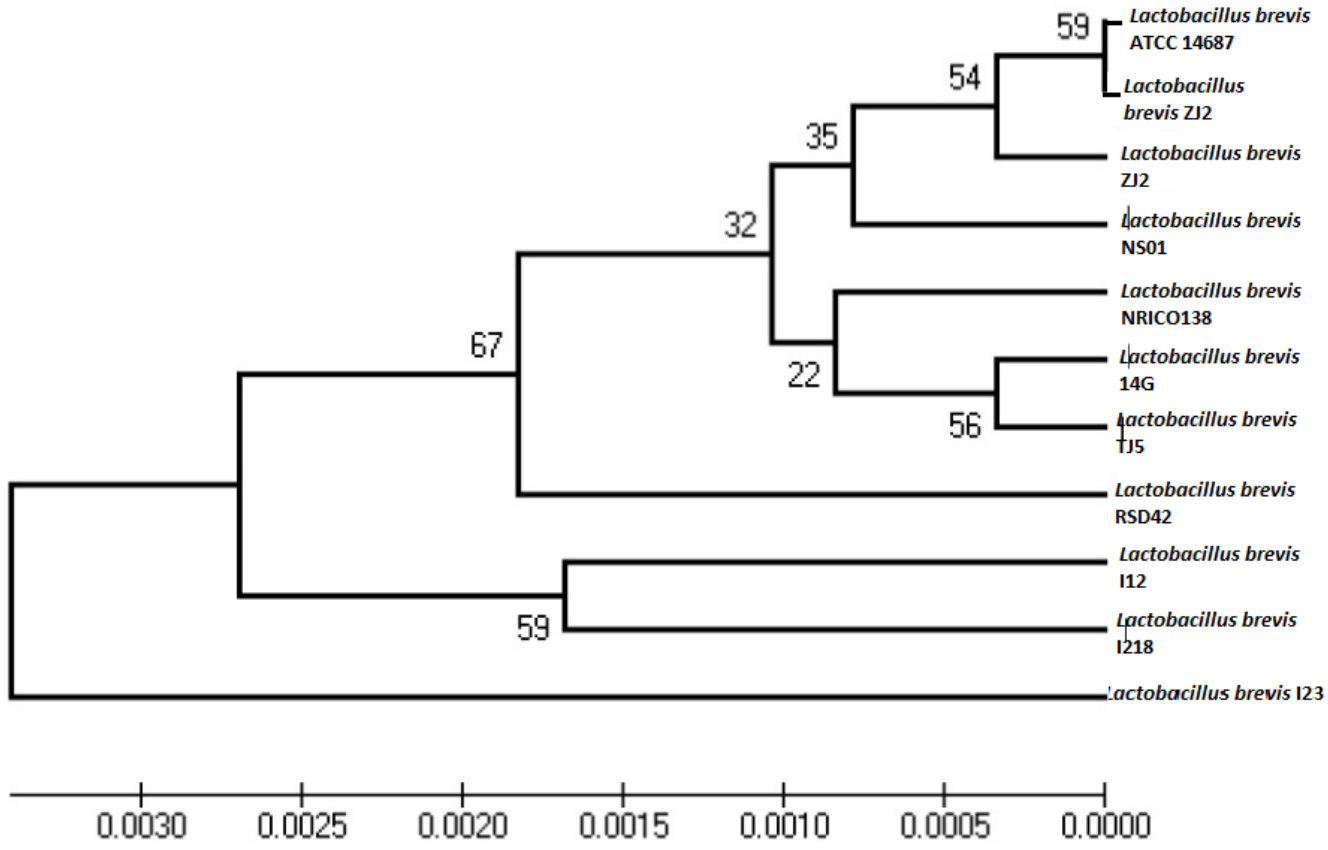

Figure 1. Phylogenetic tree showed the distance between the bacteria.

7, 9 and 10, it was selected for further study.

The isolate 5 was subjected to 16S rRNA gene sequencing and identified as *L. brevis* NS01 (NCBI, GeneBank accession number HQ702478). The phylogenetic tree was constructed using BLAST tool (Figure 1). Bacteriocin producing strains were identified on the basis of morphological, cultural characteristics and nucleotide sequence of the 16S rRNA gene and identified as *Lac. lactis* subsp. *lactis* (Stoyanova et al., 2006) and *Enterococcus faecalis, Pediococcus acidilactici* and *Pediococcus pentosaceus* (Rouse et al., 2007).

The CEP from the isolate 5 was obtained by alternative heating and cooling method. Then, it was used to determine its antimicrobial activity against 9 different food borne pathogens and the results showed that the CEP has significantly increased activity on both Gram positive and Gram negative bacteria ($P \leq 0.05$) (Figure 2). *Lac. lactis* subsp. *lactis* MC38 did not show any antimicrobial activity against the Gram negative bacteria tested. However, it is found to be active against the entire Gram positive bacteria tested (Tukel et al., 2007). Mandal et al. (2011) also proved that pediocin NV5 had strong antimicrobial efficacy against *L. mesenteroides, L. monocytogens* and *Enterococcus faecalis*.

The crude protein was purified by ammonium sulfate precipitation and dialyzed against acetate buffer (pH 5). The dialyzed protein was purified by gel filtration chromatography and then lyophilized. Since most bacteriocins have positive charge at pH near neutrality, the use of cation exchange resin is appropriate for their purification (Pingitore et al., 2007).

A quantitative study was undertaken to evaluate the activity units of the brevicin. It was observed that 12,800 and 25,600 AU/ml were obtained for CEP and purified protein, respectively for both the indicators. So, there is no difference in activity unit of brevicin among the indicators used but there was a difference in the form of protein. In contrast, Coventry et al. (1996) observed that indicators cultures showed different sensitivity to bacteriocin since the activity units were found to be different for each indicator used *L. monocytogenes* A4, *L. curvatus* and *L. seelegeri.* However, our result was coincided with

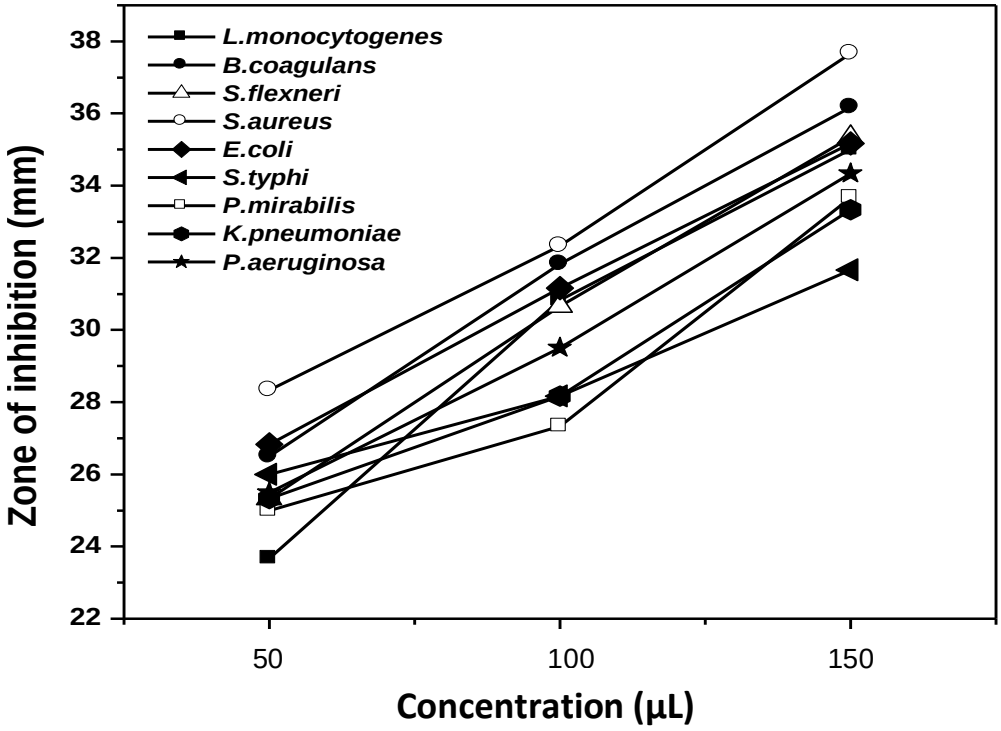

Figure 2. Antimicrobial activity of CEP of *L*. *brevis* against food spoilage organisms.

Todorov and Dicks (2005) and they reported that maximum activity of bacteriocin (12, 800 AU/ml) was recorded in MRS broth when the pH was adjusted to 5.5, 6.0 or 6.5. The protein concentration of crude and purified brevicin was found to be 1.9 and 1.3 µg ml^{-1}, respectively.

The brevicin of *L. brevis* NS01 was characterized by using different parameters and checked up their antimicrobial activity against *B. coagulans* and *S. typhi.* Among the different temperatures, 50°C for 30 min showed significantly increased activity against both the strains used ($P < 0.05$). However, the activity was retained up to 100°C (Figure 3). Like wise, the pH 5 was significantly found to be a better one and showed maximum activity, even though the considerable activity was observed from pH 3 to 9 (Figure 4).

This present study revealed that the brevicin can be used as an effective preservative for acidic foods. Similarly, Juck et al. (2010) have also reported that the antimicrobials consisting of nisin and rosemary extract was shown to be more effective in an acidic environment. The above mentioned results were correlated to the study of Tukel et al. (2007) who reported that the bacteriocin showed maximum activity at 50°C and at pH 5 but it retained its activity till 100°C for 15 min and at pH 9. The brevicin was inactivated by pepsin, proteinase K and trypsin, thereby no inhibition zone around the colonies. These results conclude that the brevicin is a proteinceous compound. The nonproteolytic enzymes like amylase, lipase, diastase and the organic solvents used did not significantly affect the antimicrobial activity of brevicin (Figure 5) and this was coincided with Naclerio (1993). Bhattacharya and Das (2010) also found similar results while treating with proteolytic and non proteolytic enzymes. The bacteriocin showed activity till 100°C for 15 min and at pH 7 and it was not activated by trypsin, lipase and catalase (Akcelik et al., 2005).

A two way ANOVA analysis of the synergistic effect of brevicin with various chemicals showed that the brevicin - EDTA combination was found to be a significant factor affecting the bacterial growth ($P \leq 0.05$) however, in all the combinations, increased activity was observed than in the chemicals used alone (Figure 6). Stevans et al. (1991) showed that treatment with nisin and chelators reduce the population of Gram negatives. The sensitivity pattern of the bacteria order was *S. aureus* > *L. monocytogens* > *E. coli* > *P. aeruginosa* > *S. typhi*. This result agrees with the study of Boziaris and Adams (1999) and they found that only EDTA and pyrophosphates were able to cause appreciable inhibition of *E. coli* by nisin. The present *in vitro* study indicates that the EDTA could be used with brevicin to increase the antimicrobial activity against Gram negatives.

Ukuku and Fett (2002) reported that the combination treatments with nisin-NaL, nisin-KS, NaL-KS and nisin

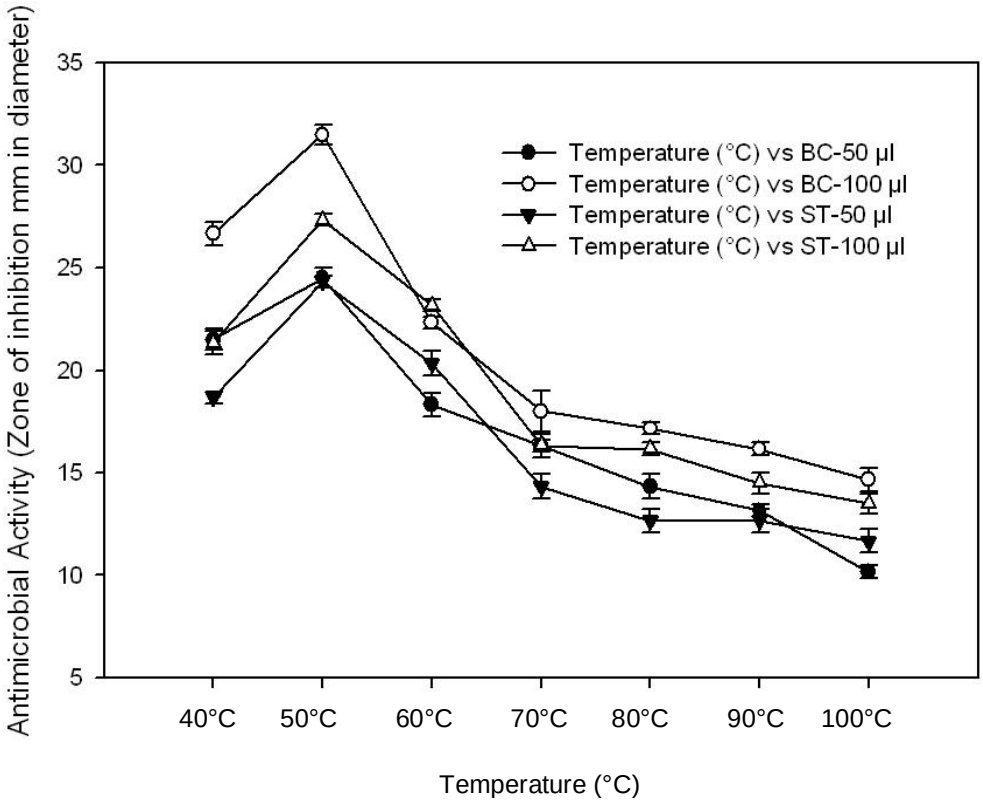

Figure 3. Effect of temperature on brevicin activity against *B. coagulans* and *S. aureus.*

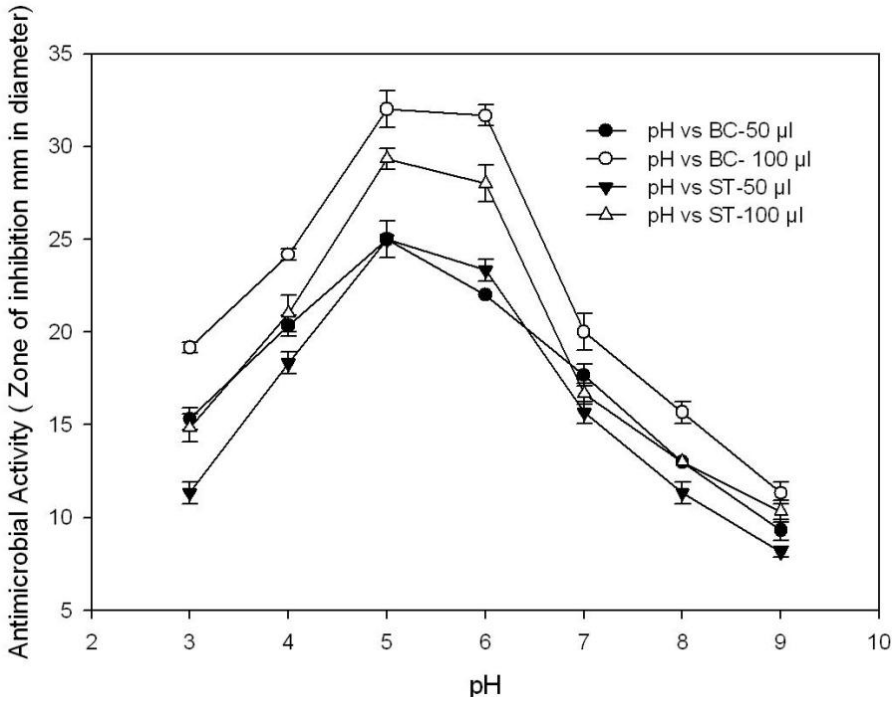

Figure 4. Effect of pH on brevicin activity against *B. coagulans* and *S. aureus.*

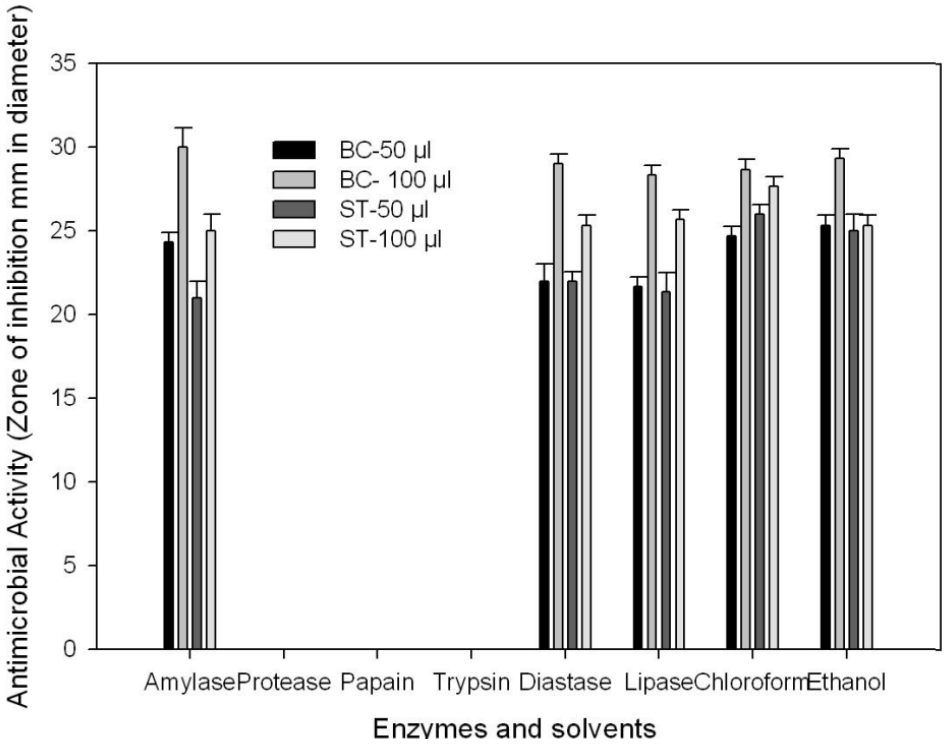

Figure 5. Effect of enzymes and organic solvents on brevicin activity against *B. coagulans* and *S. aureus.*

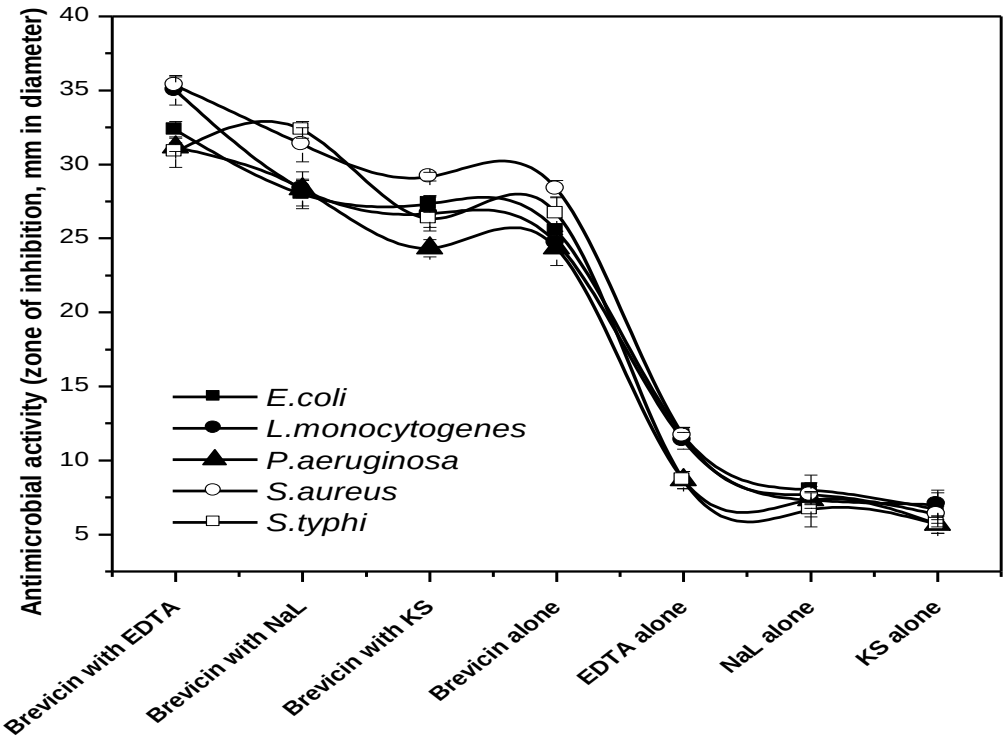

Figure 6. Effect of EDTA, Nal and KS on Brevicin activity

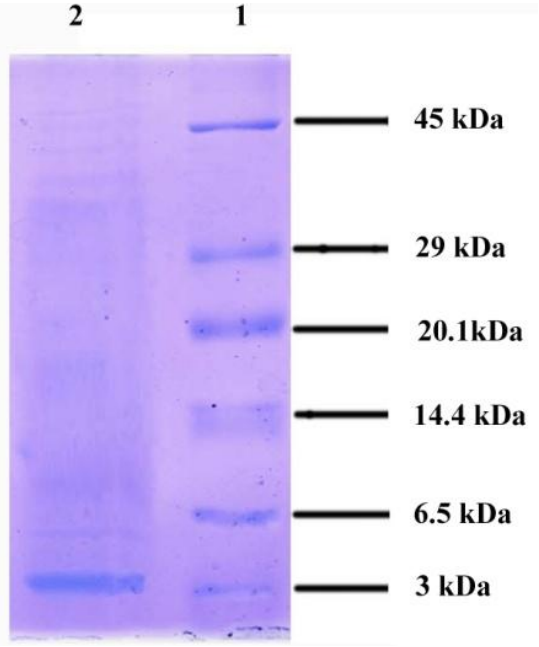

Figure 7. A protein band after SDS- PAGE of purified brevicin.

NaL-KS but not nisin-EDTA, gave significant reductions of *Salmonella* directly inoculated onto fresh cut piece of Cantaloupe. An increased inhibitory activity of bacteriocin have been observed in combination with EDTA against Gram negative bacteria (Schved et al., 1994; Helander et al., 1997) and Gram positive bacteria (Gill and Holley, 2003). Sodium lactate or sodium citrate in combination with nisin showed increased antimicrobial activity against *Arcobacter butzleri* in chicken due to their chelating effect (Long and Phillips, 2003). Sofos and Busta (1993) also proved that KS (0.02 to 0.3%) is widely used in food to inhibit yeast, mold and bacteria as these three are considered as GRAS, which may be used along with brevicin to control the microbial growth in food system.

The molecular weight of the purified protein was found to be 3.2 kDa (Figure 7); it indicates that the brevicin was low molecular weight protein. Similarly, low molecular weight of mesentericin Y 105 (Hechard et al., 1992) and bacteriocin (Bravo et al., 2009) were found to be 2.5 to 3.0 kDa and 3.5, respectively by SDS-PAGE.

The FTIR spectrum of brevicin observed at 2873 and 2947 cm^{-1} revealed the presence of aliphatic C-H stretching of fatty acids at 1565.36 and 1656.46 cm^{-1} and for the presence of amide I and amide II, respectively; at 3135.10 cm^{-1}, it showed the presence of aromatic hydrocarbon; at 3450.23 cm^{-1}, revealed the presence of primary and secondary amine (hydroxyl functionality); at 3557, exposed the presence of free hydroxyl group (Figure 8). Similarly, Teixeria et al. (2009) have reported that the FTIR spectrum of Bacteriocin like substance at 1540 and 1640 cm^{-1} corresponding to amide II and amide I, respectively (C=O,C=H stretching) and at 3250 cm^{-1} NH (stretching of proteins) were observed; at 2850-2955 cm^{-1}, characteristic of aliphatic C-H stretching of fatty acids.

NMR spectrum analysis showed certain peaks that could be raised in the τ value region 7.6 and 7.7 indicated the presence of aromatic or heteroaromatic which may contain H_2/NH group attached to ring system. The sharp narrow peak at 4.7 in the spectrum indicated that the residue may contain hydroxyl group. The peak at 4.3 gave three protons (triplet) and it explained the presence of vinyl proton with -CH_2 as neighboring group (Figure 9). This was correlated with Liu and Hansen (1990) who found resonance signal at 4.3 for vinyl protons in the residues of nisin, which is particularly suitable for proton NMR studies, because the vinyl protons in dehydro residues gave resonance signals that are easily distinguished and well separated from those of other protons in the peptide.

The presence of alkene (CH) proton was confirmed by the signal at 2.9 and the presence of β substituted aliphatic system which contains three protons were confirmed by the signal at 2.4, 2.0, 1.9 and 1.6, respectively. The six protons singlet system was arisen in the region of 1.09 and it indicated that the carbon compound might be with methyl group (CH_3-CH_3). Talarico and Dobrogosz (1989) also found out the presence of 2 three carbon compounds, β-hydroxy propion. NMR studies with deuterated methanol revealed that the compound existed as three carbon compound in a methoxy form.

The purified brevicin produced by *L. brevis* NS01 was used to extend the shelf life of pasteurized milk. Count of bacteria in plate inoculated with 1 ml of milk sample treated with 1, 3 and 5% of brevicin was shown. Better inhibition effect was observed when the milk was treated with 5% brevicin than 1 and 3%. The significant differences ($P \leq 0.05$) was observed among the brevicin treated milk samples and the control. The data obtained showed that the initial microbial load in pasteurized milk was 7.7 to 7.9 log CFU ml^{-1} but at the end of 18th day (4°C), the microbial load was only 10.5 log CFU ml^{-1} and the milk colour was retained till 18th day but this count was reached at day 6 in the control sample and the colour of the milk was slightly changed on the 9th day, the milk colour was completely changed and spoiled on 12th day. The reduction of 1.4, 2.2 and 2.8 log was observed in 1, 3 and 5% brevicin added milk, respectively (Figure 10). However, brevicin added milk was preserved up to the 18th day without any spoilage. Similarly, 0.5 and 1.7 log CFU cm^{-1} reduction of *L. monocytogens* in salmon samples packaged in film coated with 500 IU cm^{-2} of nisin for 56 days (Hudda et al., 2008) and 1 to 2 log reduction after 20 days storage of CO_2 packed cold smoked salmon treated with nisin (Nilsson et al., 1997) have been reported.

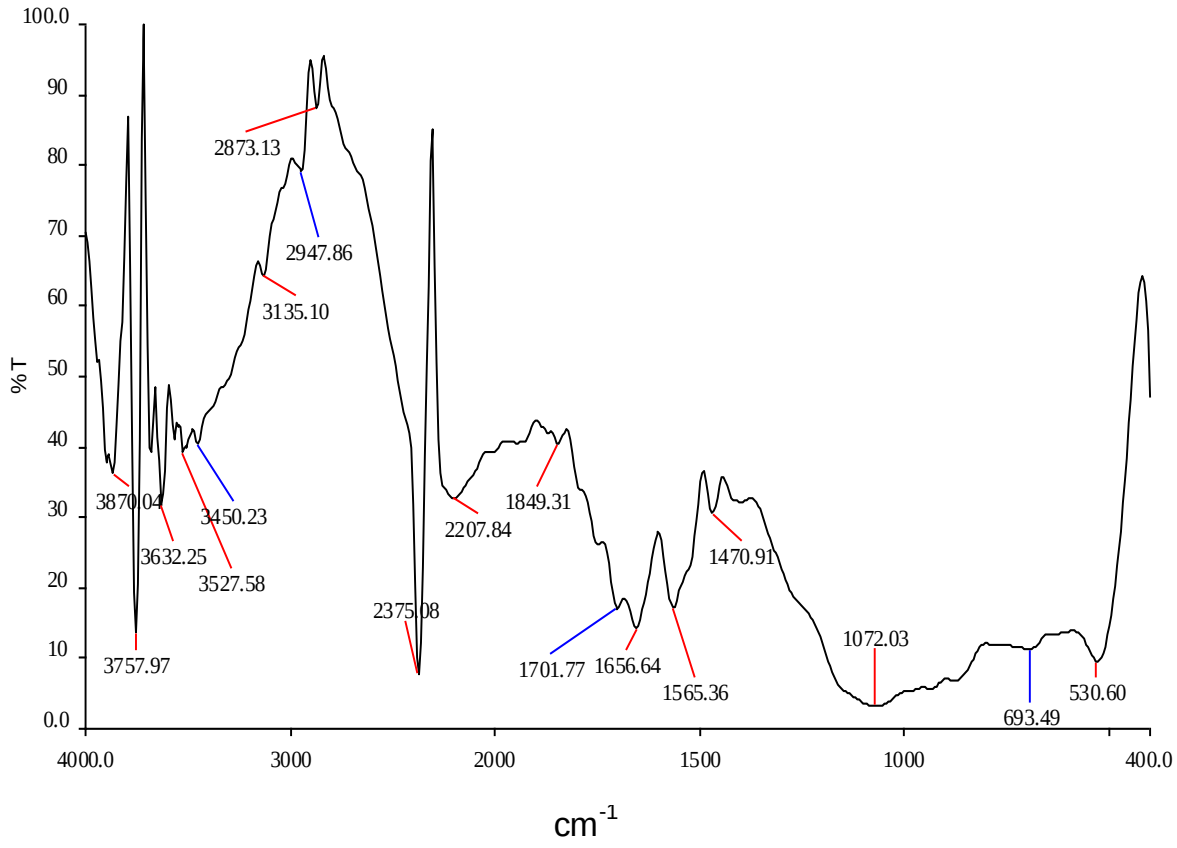

Figure 8. FTIR spectrum of purified brevicin.

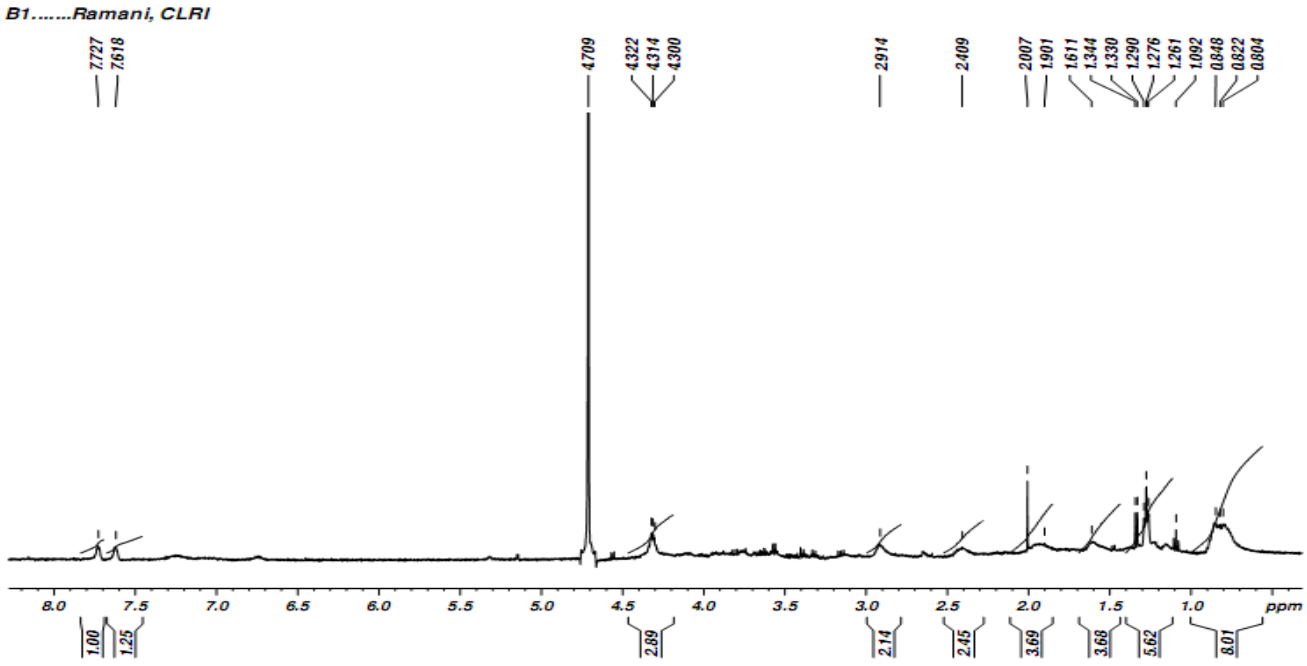

Figure 9. NMR spectrum of purified brevicin.

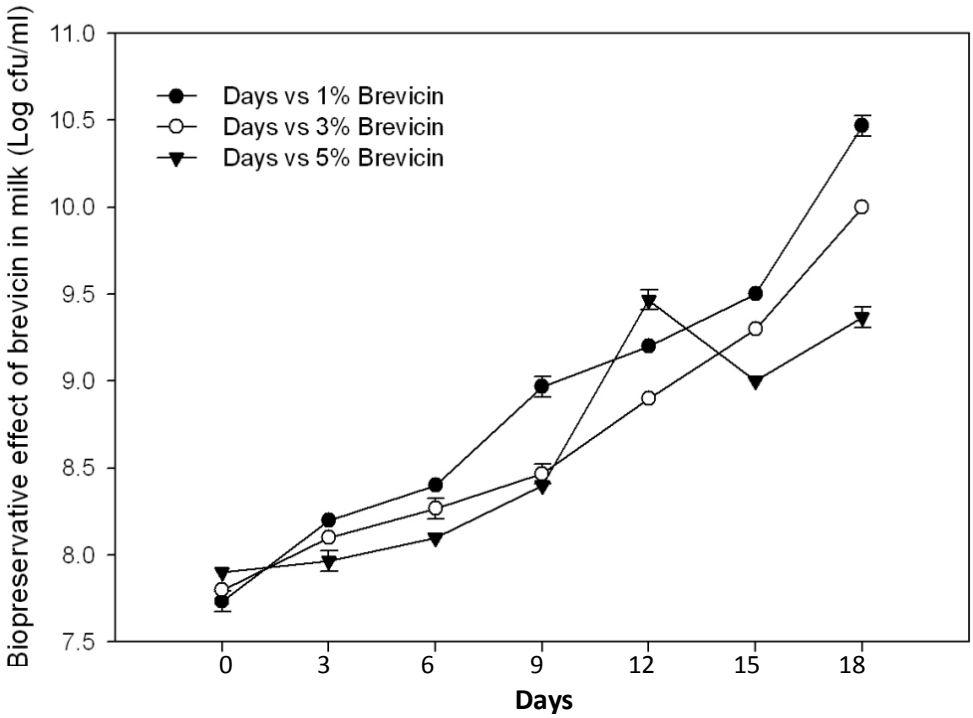

Figure 10. Preservative effect of bevicin in milk.

So far, almost all the bacteriocins produced till now possess the activity against Gram positive bacteria and very few Gram negative bacteria but in this study, a novel brevicin produced by *L. brevis* NS01 with activity against both Gram negative and positive bacteria was reported. Since mostly food borne pathogens belongs to Gram negative bacteria, the brevicin has the potential role in controlling the food borne pathogens. Therefore, it may be used as a biopreservative to control the food borne diseases.

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