ORIGINAL ARTICLE

Purification and characterization of pediocin from probiotic *Pediococcus pentosaceus* GS4, MTCC 12683

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Abstract

Pediococcus pentosaceus GS4 (MTCC 12683), a probiotic lactic acid bacterium (LAB), was found to produce bacteriocin in spent culture. Antibacterial and antagonistic potential of this bacteriocin against reference strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 25619), and *Listeria monocytogenes* (ATCC 15313) was proven by double-layer and well diffusion methods wherein nisin and ampicillin were used as positive controls. Bacteriocin in supernatant was purified and analyzed by SDS-PAGE, RP-HPLC, and circular dichroism (CD). The physico-chemical properties of purified bacteriocin were characterized being treated at different temperatures (30 to 110 °C), pH (3.0 to 12.0), with different enzymes (α-amylase, pepsin, and lysozyme), and organic solvents (hexane, ethanol, methanol, and acetone) respectively. The molar mass of bacteriocin (named pediocin GS4) was determined as 9.57 kDa. The single peak appears at the retention time of 2.403 with area amounting to 25.02% with nisin as positive control in RP-HPLC. CD analysis reveals that the compound appears to have the helix ratio of 40.2% with no beta sheet. The antibacterial activity of pediocin GS4 was optimum at 50 °C and at pH 5.0 and 7.0. The pediocin GS4 was not denatured by the treatment of amylase and lysozyme but was not active in the presence of organic solvents. This novel bacteriocin thus m ay be useful in food and health care industry.

Introduction

Bacteriocins are bacterial proteinaceous substances that inhibit the growth of similar or closely related strains of bacteria (Farkas-Himsley 1980). Bacteriocinogenic bacteria are mostly belonging to lactic acid bacteria (LAB) and are isolated from milk and milk products (Mikkili et al. 2015). Based on their source, these were categorized into several large categories including bacteriocins from Gram-positive bacteria as well as Archaea. Bacteriocins from *Escherichia coli* are known as colicins, from *Staphylococcus wameri* are termed as wamericin, and bacteriocins from LAB are designed as lantibiotics (Prema et al. 2006). Based on their activity, genetics, mechanism of killing, molecular weight, chemistry, and method of production, they are classified into three major classes, class I, class II, and class III. Nisin and lantibiotics belong to the class I bacteriocins as they are small peptide inhibitors. Class II is further divided into three groups that are classes II-a/b/c (Heng and Tagg 2006). Class II bacteriocins are small heat-stable proteins, class II-a bacteriocins have the largest subgroups, class II-b bacteriocins require two different peptides for their activity, and class II-c bacteriocins are circular bacteriocins. The bacteriocins in the group of class III are large and heat-liable protein bacteriocins. Bacteriocins isolated from *Pediococcus* spp. are designed as pediocin and belong to the type of class II-a bacteriocins with antimicrobial property. Previously, *Pediococcus* spp. and strains were used as a starter culture in the fermentation of various vegetables (Porto et al. 2017), dairy products, meat products (Meile et al. 2005; Fijan 2014; Taormina 2014), and so on. Pediocin reached at limelight due to its high anti-Listeria activity and stability at neutral pH. Gonzalez and Kunka (1987) reported that *P. acidilactici* PAC 1.0 produced a bacteriocin, designated as pediocin PA1, with molecular weight 1.65 kDa. Bhunia et al. (1987) reported that *P. acidilactici* strain H produced a bacteriocin designated as pediocin Ach, with molecular weight of 2.7 kDa which showed the antimicrobial activity against several bacterial species. Many strain-specific pediocinogenic *Pediococcus* spp. were isolated and reported with different characteristics of pediocins from different studies, like pediocin JD (*P. acidilactici* SJ-1), pediocin 5 (*P. acidilactici*

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UL5), pediocin A (*P. pentosaceus* FBB-61), pediocin N5p (*P. pentosaceus*), and pediocin ST18 (*P. pentosaceus*) (Porto et al. 2017). The present study reports about pediocin GS4 from *P. pentosaceus* GS4.

Our research group has previously isolated and identified *P. pentosaceus* GS4 from the well-known Indian fermented food *Khadi* (Sukumar and Ghosh 2010a, b) and the same strain has been deposited in GenBank (NCBI HM044322) and Microbial Type Culture Collection (MTCC), India, and has received the deposited accession number, MTCC 12683. *P. pentosaceus* GS4 has probiotic potential being acid tolerant, bile salt tolerant, lactic acid homofermenter, β-galactosidase producer, vancomycin resistant, assimilates cholesterol, anti-oxidative (Sukumar and Ghosh 2011), and also exerts its bactericidal (antibacterial) effect (Sukumar and Ghosh 2010a, b). *P. pentosaceus* GS4 has bio-hydrogenation property and can produce CLA (conjugated linoleic acid) from diverse source of vegetable oils (Dubey et al. 2012) which has demonstrable role to play in the mitigation of induced toxicity in the liver, kidney, and intestine and GS4 demonstrates that it is a safe and nontoxic probiotic strain (Dubey et al. 2015). The probiotic GS4 is also good to control the induced colon carcinogenesis in mice inducing apoptosis (Dubey et al. 2016). Besides, study on viability of *P. pentosaceus* GS4, in simulated gastric condition, showed maximum survivability and resistance to processing stress and further viability and stability may be achieved in the presence of protective agents such as lactose, ascorbic acid, and inulin (Bagad et al. 2012). Following this, we studied also the survival rate of probiotic strains of *P. pentosaceus* GS4, *P. pentosaceus* GS17, and *Lactobacillus gasseri* and were evaluated recently by using the method of lyophilization with individual and in combination of excipients, gradually up to 120 days on storage at 4 ± 1 °C. The effect of freeze drying and excipients on probiotic properties such as lactic acid production, antimicrobial effect, adherence to epithelial cells, growth of the probiotics, and survivability were examined during the storage conditions (Bagad et al. 2017).

Previous research works primarily showed that the antagonistic effect of *P. pentosaceus* GS4 against the pathogenic microbes like *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Staphylococcus aureus* resembles to their intrinsic properties primarily due to secretion of antimicrobial compound including lactic acid (Daeschel and Klaenhammer 1985; Lee et al. 2007; Sukumar and Ghosh 2010a, b). The present study is therefore designed to report the antagonistic ability of *P. pentosaceus* GS4, due to the elaboration of antibacterial protein molecule, its purification, demonstration of its biological activity being treated with different physicochemical components, and analytical characterization

using SDS-PAGE, circular dichroism (CD), and RP-HPLC, respectively.

Materials and methods

Bacterial strains

The laboratory probiotic strain *Pediococcus pentosaceus* GS4 (MTCC 12683), isolated from Indian fermented food *Khadi*, identified by 16S rRNA sequencing (accession number in GenBank: NCBI HM044322 (GS4)), was used in this study. Besides, four reference strains like *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 15313), *Pseudomonas aeruginosa* (ATCC 25619), *Escherichia coli* (ATCC 25922), and a LAB strain, *Lactobacillus gasseri* (ATCC 19992), obtained from Microbiologics, Medimark Europe, France, were also used.

In vitro antagonistic activity of *P. pentosaceus* GS4

Antagonistic activity was determined by the double-layer method (Maia et al. 2001). Overnight grown culture of *P. pentosaceus* GS4 was inoculated (10^4cfu/spot) on De Mann Rogosa Sharpe (MRS) (Hi Media Laboratory, Pvt. Ltd., India) agar by spot inoculation and incubated at 37 °C for 48 h. After incubation, the cells were killed by exposure of each dish to 4 mL chloroform for 20 min. The chloroform residue was allowed to evaporate and the agar in each dish was overlaid with 10 mL of brain-heart infusion agar (Hi Media Laboratory, Pvt. Ltd., India) (0.75%) containing 0.2 mL of inoculum of reference organism (2×10^9 cfu/mL). After 48 h of incubation, the plates were evaluated for the presence of zones of inhibition (ZOI). The final ZOI diameter corresponded to the difference between the total inhibition zone and the diameter of the colony. The experiment was repeated thrice with each reading representing the mean of 12 observations. Parallelly, reference bacterial strains (2×10^9 cfu/mL) cultured without *P. pentosaceus* GS4 intervention were treated as negative controls.

Acidifying potential of *P. pentosaceus* GS4 in comparison with *L. gasseri* (ATCC 19992)

Acidifying activities were studied by inoculating 50 mL of MRS broth with 1% of overnight grown culture of *P. pentosaceus* GS4 and *L. gasseri* individually and were incubated at 37 °C for 24 h. The change in pH (ΔpH) was used as a measure of acidification. The pH was measured every 2 h until 24 h using a pH meter (Orion Smart Check Meter, Thermon Electron Corporation, Beverly, USA). The acidification values

were expressed as ΔpH and were calculated using the equation below:

$$\Delta pH = pH_t - pH_0,$$

where pH_t is the pH of the culture at time t and pH_0 is the pH at zero time.

Ayad et al. (2004) described the acidifying activity of cultures as fast, medium, or slow acidifying when a ΔpH of 0.4 U (pH units) was achieved after 3, 3–5, and > 5 h, respectively.

Preparation of cell-free supernatant of *P. pentosaceus* GS4

P. pentosaceus GS4 (1% overnight grown bacterial culture) was inoculated into 100 mL of sterile MRS broth (pH 6.5) and was incubated at 37 °C for 48 h. The culture was centrifuged at 12,180g for 15 min at 4 °C. The supernatant was collected and was divided into two parts: a part was adjusted to pH 7.0 using 1 M NaOH (neutralized) and other part was kept as such (fresh). Both parts were then filter-sterilized using 0.45-μm membrane syringe filter (Sartorius).

Inhibitory activity of CFS of *P. pentosaceus* GS4

Inhibitory activity of the fresh and neutralized CFS (fCFS and nCFS) of *P. pentosaceus* GS4 was tested on the reference strains (*S. aureus*, *E. coli*, *P. aeruginosa*, and *L. monocytogenes*) in order to determine the relative zone of inhibition (ZOI) by the agar well diffusion method (Lasta et al. 2008) either due to lactic acid and bacteriocin or bacteriocin only, the minimum volume (μL) and minimum hour required inhibiting the growth of the reference strains.

Antibacterial activity of fCFS and nCFS of *P. pentosaceus* GS4 was determined by the agar well diffusion method (Lasta et al. 2008). In brief, reference bacterial culture (*S. aureus*, *E. coli*, *P. aeruginosa*, and *L. monocytogenes*) of the exponential phase was inoculated onto the Mueller-Hinton agar (MHA) (Hi Media Laboratory, Pvt. Ltd., India) plate surface using a sterile cotton swab. A well of 8-mm diameter was cut aseptically onto the cultured agar using a sterile cork borer. A volume of 100 μL CFS (fresh and neutralized) was then instilled into individual well and was incubated at 37 °C for 24 h (Lasta et al. 2008).

Reference bacterial strains (*S. aureus*, *E. coli*, *P. aeruginosa*, and *L. monocytogenes*) were treated with different volumes of neutralized CFS over time to determine the limit of inhibition. Briefly, different volumes of nCFS (50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 μL) were treated with 10^6–10^8 CFU/mL of reference bacterial culture of the exponential phase for 0, 1, 2, 3, 4, 5, 6, 7, and 8 h at 37 °C. A 100 μL of thus treated culture broths of every hour and from every volume were subcultured onto MHA and were

incubated for 18–24 h at 37 °C. All the plates were then examined and viable colonies were enumerated. Corresponding untreated reference bacterial cultures of the exponential phase were used as negative controls. Difference in enumerated viable colonies between test (treated) and control (untreated) was calculated and compared

Isolation and partial purification of nCFS by ammonium sulfate precipitation and dialysis

For the isolation and partial purification of antimicrobial compound, *P. pentosaceus* GS4 (1% overnight grown bacterial culture) was inoculated into 100 mL of MRS broth (pH 6.5) and was incubated at 37 °C for 48 h. The cell-free supernatant (CFS) obtained by centrifugation (12,180g for 15 min at 4 °C) was adjusted to pH 7.0 using 1 M NaOH. A 26.67 g of ammonium sulfate was added to attain precipitation at 45% saturation (Aktypis et al. 1998) and was kept to settle at 4 °C for 24 h. The precipitated protein was recovered by centrifugation at 12,180g for 10 min at 4 °C and was dissolved in 10 mL 20 mM phosphate buffer (PB) (0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4) (pH 6.8). The dissolved protein was dialyzed using 1K MWCO (molecular weight cutoff) at 4 °C for overnight with frequent changes of phosphate buffer (pH 6.8) (Sambrook and Russell 2001). The protein concentration was measured at every step of isolation and partial purification by Lowry's method using bovine serum albumin as protein standard (Lowry et al. 1951). This dialyzed protein, pediocin GS4, was used for further experiments and analysis.

Determination of antibacterial activity of pediocin GS4

Antimicrobial activity of pediocin GS4 was determined by the agar well diffusion method (Lasta et al. 2008) using reference strains of *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, and *E. coli*. Reference strains were subcultured onto Mueller-Hinton agar (pH 6.5) at the log phase (approx.10^9 cfu/mL) using the sterile swab. Wells were made puncturing agar using the incinerated cork borer of 8-mm diameter. A 100 μL (80 μg) of dialyzed pediocin GS4 was instilled into wells. Nisin (100 μg/100 μL) and ampicillin (10 μg/100 μL) were used as positive controls and sterile phosphate buffer saline (PBS) (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4) as negative control. The antimicrobial activities were determined by the measurement of the diameter (mm) of zone of inhibition (ZOI) around the wells after incubation at 37 °C for 24 h and were compared between positive and negative controls. The experiment was performed in duplicates and each of the readings was taken by two observers and the mean was calculated.

Determination of molar mass of pediocin GS4 by SDS-PAGE

The molar mass of the pediocin GS4 was determined by SDS-PAGE (16%) (Sandbrook and Russell 2001). An 8 mL of 16% acrylamide separating gel was added to the glass assembly and over layered with distilled water (to remove the un-polymerized acrylamide) and allowed for solidification. A 5 mL of 4% acrylamide stacking gel was layered on the separating gel. Protein molecular marker supplied by Genei (India) and bovine serum albumin (Hi Media, India) were used as a reference in this study. The dialyzed pediocin GS4 and the protein marker were subjected to heat treatment at 90 °C for 90 s after adding equal volume of sample buffer. The heat-treated samples were loaded into the wells and electrophoresis was performed for a period of 1 h at 50 V (cold condition). The separated proteins were stained with staining solution containing Coomasie Brilliant Blue for 4 h on a rocker at low speed. The bands were visualized after de-staining overnight. The molar mass of the pediocin was determined from the standard graph of log of molecular weight versus distance (supplement).

Purity analysis of pediocin GS4 by HPLC

The dialyzed pediocin GS4 was further purified by RP-HPLC (reverse phase, HPLC). Pediocin GS4 as well as nisin (commercial bacteriocin) was diluted fivefold with PBS (pH 6.8) before injection into the HPLC apparatus (Waters, USA). They were chromatographed on a 150×4.6-mm Perkin Elmer 200 series (C-18) reverse phase HPLC column equilibrated with 0.1% trifluoroacetic acid (v/v)/acetonitrile at a flow rate of 1 mL/min, with the absorbance (220 nm) using a UV detector (Kwon and Kim 1994). The retention time and the area under the peaks were recorded to analyze the results.

Structure analysis of pediocin GS4 by CD spectroscopy

CD spectroscopy of pediocin was recorded at Jasco (J-715) spectrophotometer (Jasco, Tokyo, Japan) at a protein concentration of 0.8 mg/mL dissolved in 0.02 M PBS, at pH 7.4 with a 1.0 cm of path length. The pediocin GS4 samples were scanned five times at the rate of 50 nm/min with a bandwidth of 0.5 nm and a response time of 1 s over the wavelength range from 200 to 250 nm. The data were averaged and the spectrum of a sample-free control was subtracted. The α-helical content of the peptides was determined by the application of the spectral fitting method control/LL (CD-5 pdf), CDSSTR (CD-5 pdf), and SELCON3 (CD-5 pdf), in CD pro package (CD-5 pdf). The single-point method was also used. In this method, the helicity is calculated from the mean residual ellipticity at 222 nm (CD-5 pdf).

Determination of the effect of enzymes on purified pediocin GS4

The antibacterial activity of pediocin from *P. pentosaceus* GS4 was determined after treatment with different proteolytic and non-proteolytic enzymes (pepsin, α-amylase, and lysozyme). Pediocin GS4 and nisin (control) were treated with the above enzymes at a final concentration of 1 mg/mL. The enzyme-treated pediocin GS4 and nisin were then subjected for incubation at 30 °C for 1 h followed by 1 h of incubation and samples were heated at 50 °C for 7 min to inactivate the effect of enzymes (Jimenez-Diaz et al. 1993). These samples were then subjected for bioassay to examine the antibacterial activity against different bacterial strains by the agar well diffusion method.

Determination of the effect of different pH on pediocin GS4

To determine the effect of pH, the pediocin GS4 was treated with various pH (3.0, 5.0, 7.0, 9.0, and 12.0) using sterile buffers (pH 3–5, potassium hydrogen phthalate solution; pH 6–8, potassium dihydrogen phosphate solution; for pH 9–10, solution of sodium tetraborate; pH 11–12, disodium hydrogen phosphate and sodium hydroxide solution) and was then subjected for incubation at 35 °C for 1 h. After incubation, samples were adjusted to pH 7.0 using 0.1 M of NaOH and 0.1 M of HCl. The pH-adjusted pediocin GS4 was then examined against different reference bacterial strains for determining antibacterial activity (Albano et al. 2007).

Determination the effect of different temperatures on pediocin GS4

The bacteriocin activity of pediocin GS4 was determined on the treatment with different temperatures (30, 40, 50, 60, 70, 90, and 110 °C) for both 15 and 30 min respectively. After treatment, the pediocin GS4 samples were kept at room temperature for 30 min and then were examined the bioassay against different reference strains by the agar well diffusion method (Todorov and Dicks 2006).

Determination the effect of different organic solvents on pediocin GS4

Antibacterial activity of pediocin GS4 was determined on treatment with different organic solvents such as, hexane, ethanol, methanol, and acetone. Equal volume of each organic solvent was mixed with pediocin GS4 (0.8 mg/mL) and was then subjected to incubation at 4 °C for 1 h. After incubation, the organic solvents were removed from the mixture by evaporation at room temperature (~ 28 °C) and then were

examined for bioassay (ten Brink et al. 1994) against different reference strains by the agar well diffusion method.

Statistical analysis

Results obtained from different sets of experiments were analyzed statistically by calculating the mean with standard deviation (SD) and standard error (SE) respectively wherever necessary from a minimum of three observations. All the statistical analysis was carried out using GraphPad Prism version 5.0 (GraphPad software) at significance level $p \leq 0.05$.

Results

In vitro antagonistic activity of *P. pentosaceus* GS4

To have an impact on the colonic microbiota, it is important for the probiotic strains to show antagonism against pathogenic bacteria via antimicrobial production or competitive exclusion. In vitro antagonistic activity of *P. pentosaceus* GS4 investigated using the double-layer method showed maximum zone of inhibition (ZOI) against *S. aureus*, *P. aeruginosa*, and minimal with *E. coli* and *L. monocytogenes*. The average ZOI from triplicate experiments were 35.75 ± 1.0, 26.25 ± 1.3, 23.63 ± 0.6, and 10.50 ± 0.4 (mm) for *S. aureus*, *P. aeruginosa*, *E. coli*, and *L. monocytogenes* respectively (Fig. 1). The antagonistic attribute may be a reflection of production of lactic acid and bacteriocin-like protein.

Comparison of the acidifying potential of *P. pentosaceus* GS4 with that of *L. gasseri* (ATCC 19992)

Slow acidifying activity is one of the key characteristics of being an ideal probiotic. Acidifying activity of LAB lowers

the pH of the medium, lowers the pH of the digestive tract, which creates an unfavorable environment for the pathogens to grow. The acidifying activity of *P. pentosaceus* GS4 is shown in Fig. 2, in comparison with *L. gasseri*. Both strains showed significantly lowering of pH with the final pH at 24 h being 4.35 and 4.15 for *P. pentosaceus* GS4 and *L. gasseri* respectively and hence have the potential to inhibit the coliform bacteria by slow acidification.

### Inhibitory activity of CFS of *P. pentosaceus* GS4

The CFS of *P. pentosaceus* GS4 demonstrated the antibacterial activity with and without neutralization. Not-neutralized (fresh) fCFS (which contains both lactic acid and bacteriocin) showed the ZOI against Gram-positive strains; *S. aureus* and *L. monocytogenes* are 25.03 ± 2.1 mm and 17.23 ± 1.0 mm respectively, while against Gram-negative strains, *P. aeruginosa* and *E. coli* were 18.83 ± 1.0 mm and 12.83 ± 0.5 mm respectively. The comparison of antibacterial potential of *P. pentosaceus* GS4 with reference LAB strain, *L. gasseri*, is shown in Table 1. Neutralized CFS (nCFS) (which contains bacteriocin) showed reduced ZOI. The ZOI was found reduced by 31.4% in *S. aureus*, 20.3% in *L. monocytogenes*, 25.3% in *P. aeruginosa*, and no reduction in *E. coli* respectively (Table 1). The antibacterial activity shown over acid neutralization thus demonstrates the presence of bacteriocin-like molecules in nCFS.

Inhibitory activity of nCFS of *P. pentosaceus* GS4

Treatment with nCFS of *P. pentosaceus* GS4 showed variable degree of inhibitory activity against the reference strains studied through the viable count method.

The reduction in viable count of *S. aureus* after exposure to nCFS was observed. The cell count was decreased by 2 log values at the end of the first hour of treatment and was

Fig. 1 In vitro antagonistic activity of *P. pentosaceus* GS4 against reference bacterial strains (*S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 15313), *P. aeruginosa* (ATCC 25619), and *E. coli* (ATCC 25922))
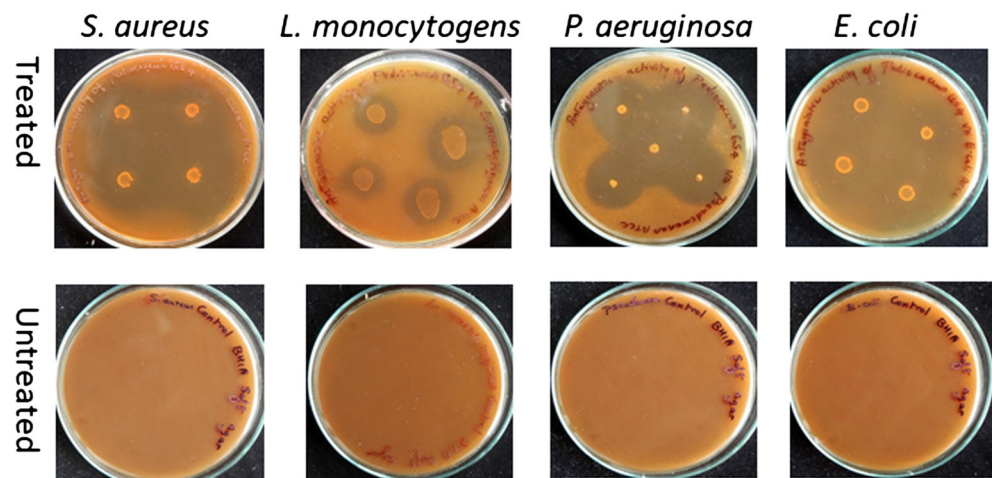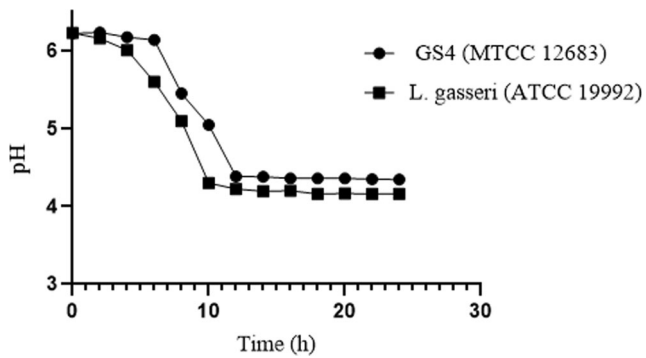
Fig. 2 Acidifying potential of *P. pentosaceus* GS4 in comparison with *L. gasseri* (ATCC 19992)

gradually decreased by sevenfold from the first hour to the third hour (Fig. 3). In *P. aeruginosa*, the cell count was reduced to half of the initial value at the end of the first hour of treatment. The cell count was reduced to 1/4th between the 1st and the 2nd hour of treatment. And a sevenfold reduction was observed between the 2nd and the 3rd hour of treatment. An exponential pattern of reduction in the viable count was observed in *P. aeruginosa* (Fig. 3). *E. coli* was gradually reduced during the first 4 h of the treatment and there was a sixfold decrease in viable count by the end of the 4th hour when compared to the viable cells at the beginning of the treatment (Fig. 3). Likely, the viable count of *L. monocytogenes* showed reduction by fourfold after 1 h of treatment with the nCFS. A difference of 2 logs was observed between the viable cell count at the 0th hour and the 4th hour (Fig. 3).

It was revealed that variable quantities (μL) of nCFS were required for complete inhibition of viability of reference strains in use at the elapse of variable hours of treatment. An aliquot of 300 μL of nCFS showed no growth after 4 h of treatment against *S. aureus* when compared to control (without treatment). Inhibition in growth was observed after 4 h of

treatment for *P. aeruginosa* with 350 μL. However, 5 h of treatment with higher volumes of nCFS was required for *E. coli* (450 μL) and *L. monocytogenes* (500 μL) respectively (Fig. 3).

Isolation and partial purification of pediocin GS4 by ammonium sulfate precipitation and dialysis

The nCFS of *P. pentosaceus* GS4 (3.78 mg/mL) was precipitated with ammonium sulfate at 45% saturation (1.64 mg/mL) followed by dialysis against 20 mM PB (pH 6.8). The protein concentration of the dialyzed sample was determined as 0.8 mg/mL. This preparation of nCFS is referred as pediocin GS4 which was further analyzed and assayed for the antibacterial activity following the agar well diffusion method.

Determination of antibacterial activity of pediocin GS4

The pediocin GS4 showed the maximum and minimum antibacterial activity against *S. aureus* (ATCC 25923) and *L. monocytogenes* (ATCC 15313) with ZOI of 24.25 ± 0.8 mm and 15.25 ± 1.2 mm respectively (Table 2). Sterile PBS (negative control) did not show any zone of inhibition.

Determination of molar mass of pediocin GS4 by SDS-PAGE

The molar mass of pediocin GS4 was determined from 16% SDS-PAGE by plotting a standard graph (log of molar mass versus distance) as 9.57 kDa (supplement). The protein bands on the gel stained with Coomasie Brilliant Blue are shown in Fig. 4.

Table 1 Antimicrobial activity of fresh and neutralized CFS of *P. pentosaceus* GS4

| Strains	Inhibition zone diameter (mm) against the indicator strain			
	S. aureus	*L. monocytogenes*	*P. aeruginosa*	*E. coli*
Antimicrobial activity of fresh CFS (fCFS)				
P. pentosaceus GS4	25.03 ± 2.1	17.23 ± 1.0	18.83 ± 1.0	12.83 ± 0.5
L. gasseri (ATCC 19992)	20.50 ± 0.1	21.00 ± 1.0	20.00 ± 0.0	No ZOI
Positive control (ampicillin)	43.00 ± 2.6	38.00 ± 0.4	19.67 ± 1.0	30.67 ± 0.3
Negative control (blank broth)	No ZOI			
Antimicrobial activity of neutralized CFS (nCFS)				
P. pentosaceus GS4	17.53 ± 0.8	13.73 ± 0.5	14.06 ± 0.5	13.20 ± 0.7
L. gasseri (ATCC 19992)	13.58 ± 0.5	No ZOI	13.56 ± 0.9	No ZOI
Positive control (ampicillin)	41.00 ± 0.6	34.50 ± 0.5	19.00 ± 1.0	23.08 ± 0.0
Negative control (blank broth)	No ZOI			
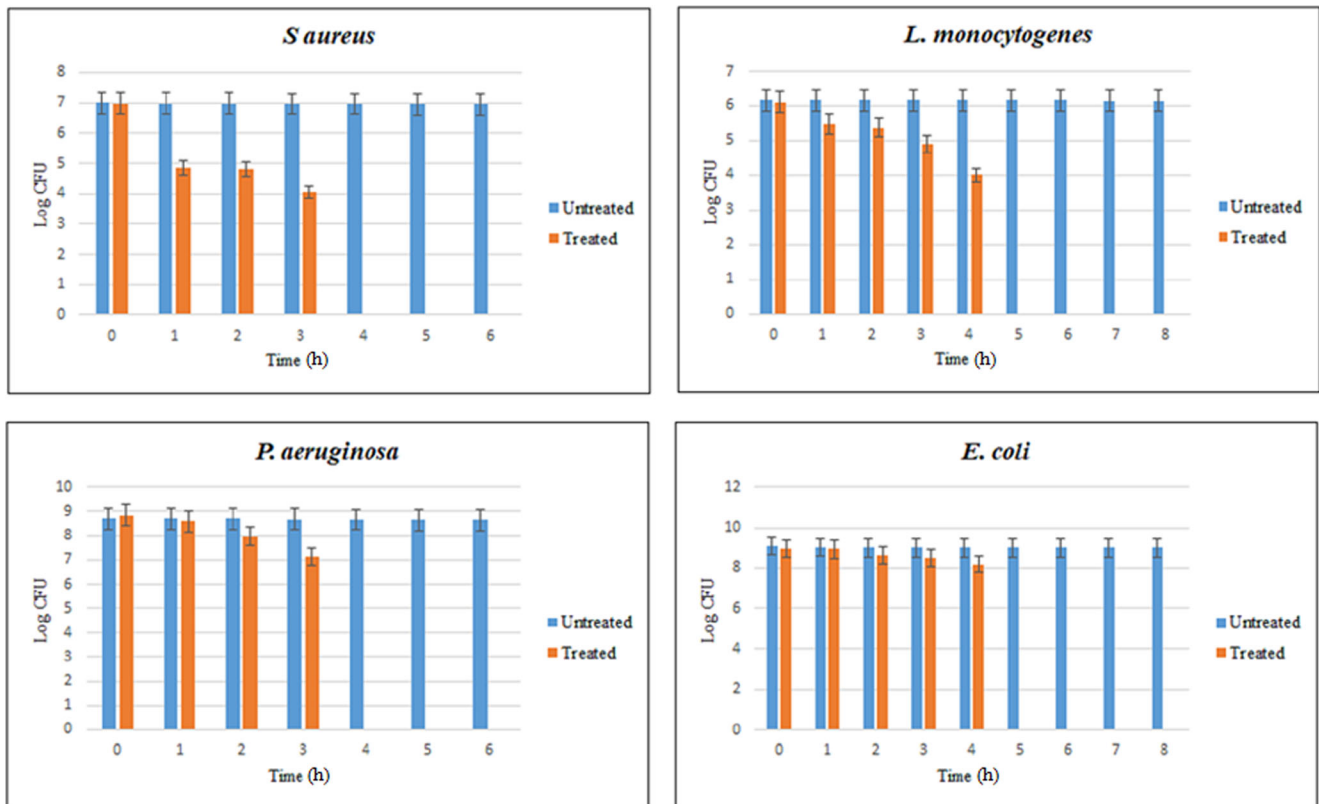
Fig. 3 Viability of reference bacterial cells with the treatment of neutralized CFS of *P. pentosaceus* GS4 over time

Purity analysis of pediocin GS4 by RP-HPLC

The dialyzed pediocin GS4 was further purified by subjecting it to RP-HPLC. A single peak was observed. A single peak indicates the homogeneity and purity of the sample. It was generally showed in previous studies that the maximum peak obtained at different time in semi preparative RP-HPLC may be because of its incubation time, medium composition, and purification speed. The major peak with the reference nisin was observed at retention time of 2.43 min and with the area of the peak being 65.20%. The retention time for the major peak of the dialyzed protein appeared at 2.403 min with the percentage of area amounting to 25.02%. This infers that the overlay of the chromatogram showed overlap

of the peaks indicating the identical nature of the biomolecules and this shows the purity of isolated protein sample (Kwon and Kim 1994). The chromatogram of RP-HPLC of the reference and the sample (pediocin GS4) is shown in Fig. 5.

Analysis by CD spectroscopy

The CD data was analyzed with the single-point method at 222 nm and with three other different methods. The spectrum method gave the α-helical structure of pediocin GS4 shown in Fig. 6. The secondary structure was determined by CD spectroscopy in the "far-UV" spectral region (190–250 nm). At these wavelengths, the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded

Table 2 Antibacterial activity of pediocin GS4 against bacterial reference strains (mean ZOI in mm)

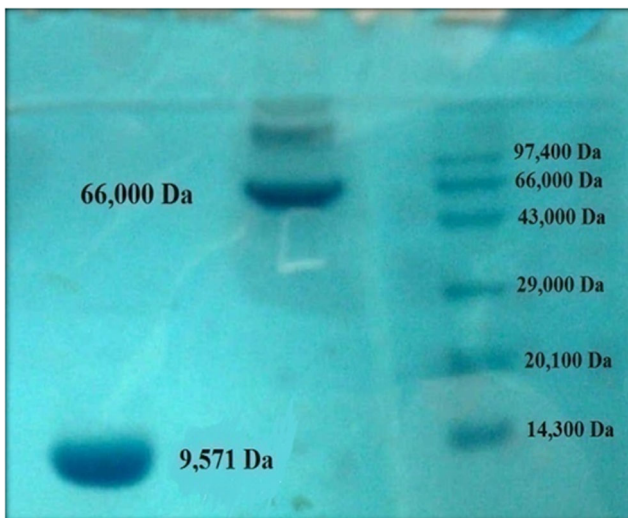|	*Staphylococcus aureus*	*Listeria monocytogenes*	*Pseudomonas aeruginosa*	*Escherichia coli*
Pediocin GS4(80 μg/100 μL)	24.25 ± 0.8	15.25 ± 1.2	17.25 ± 0.7	16.25 ± 0.8
Nisin(100 μg/100 μL)	21.5 ± 0.5	No zone	No zone	No zone
Ampicillin (10 μg/100 μL)	41.9 ± 0.5	34.5 ± 0.8	19.0 ± 0.5	23.08 ± 0.7
Sterile PBS (negative control)	No ZOI			

Fig. 4 Determination of molecular weight of pediocin GS4 by SDS-PAGE lane 1: pediocin GS4 (9.57 kDa); lane 2: bovine serum albumin (BSA); lane3: molecular marker (14,300–97,400 Da) (Genei, India)

environment. As per the CD analysis of purified pediocin GS4, the compound appears to have the helix ratio of 40.2% with no beta sheet, hence unfolded with turn (20.7%) and random of 39.1% respectively (Fig. 6).

Effect of enzymes on the activity of pediocin GS4

The effect of various enzymes such as α-amylase (1 mg/mL), pepsin (1 mg/mL), and lysozyme (1 mg/mL) on the bacteriocin activity of pediocin GS4 was determined by measuring zone of inhibition appeared after 24 h of incubation at 37 °C. It was observed that the antibacterial activity was retained among the α-amylase- and lysozyme-treated pediocin GS4 samples only against *S. aureus*, *E. coli*, and *L. monocytogenes* with reduced ZOI, while pediocin GS4 got completely denatured after treatment with pepsin (Table 3).

Effect of different pH on pediocin GS4

The antibacterial activity of the bacteriocin may be changed at different pH. We have found that pediocin GS4 retains its antibacterial property at pH 5.0 and pH 7.0 (Table 4). Pediocin GS4 demonstrated the maximum antibacterial activity at pH 7.0 against *P. aeruginosa* (ZOI, 27.50 ± 0.8 mm), while the minimum ZOI (19.00 ± 0.4 mm) against *S. aureus*. The bactericidal activity of pediocin GS4 was completely inactivated at pH 3.0, 9.0, and 12.0 and showed no zone of inhibition.
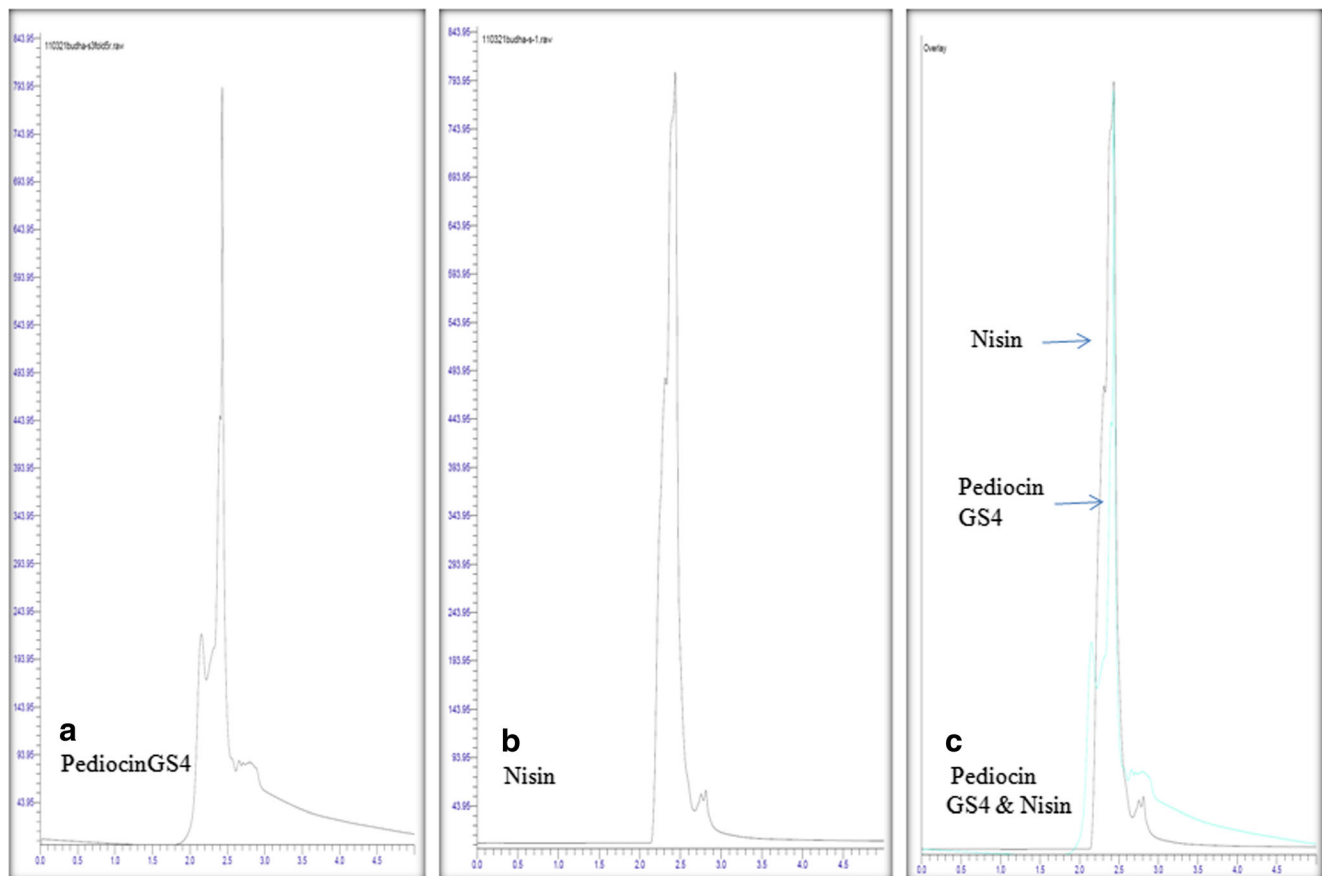

Fig. 5 Display of HPLC-purified pediocin GS4. **a** and **b** show the peak of the pediocin GS4 and standard (nisin) at fivefold dilution with PBS against 0.1% TFA (trifluoroacetic acid) in acetonitrile. The flow rate was 1 mL/min. **c** shows the combination of both **a** and **b**
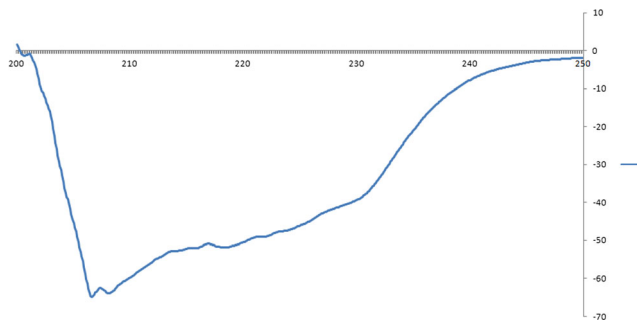
Fig. 6 The secondary structure of pediocin GS4 determined by CD spectroscopy in the "far-UV" spectral region (190–250 nm)

Effect of different temperatures on the activity of pediocin GS4

The effect of temperatures (30, 40, 50, 60, 70, 90, 100, and 110 °C) on the antibacterial activity of pediocin GS4 was determined by measuring the ZOI appeared after 24 h of incubation at 37 °C against reference strains. It was observed that the pediocin GS4 was found to be heat sensitive as it could retain the antibacterial potential being treated between 30 and 50 °C (Table 5) with different duration. *P. aeruginosa* was found sensitive to pediocin GS4 treated with 30 °C for 15 and 30 min and showed higher ZOI (34.00 ± 0.8 mm and 29.00 ± 0.6 mm), whereas the minimum antibacterial activity was appeared against *E. coli* was 16.00 ± 0.9 mm and 11.50 ± 0.7 mm respectively with 50 °C-treated pediocin GS4 (Table 5). Pediocin GS4 treated with 30–50 °C for 15 and 30 min showed its antibacterial activity against *S. aureus* and *L. monocytogenes* (Table 5). The pediocin GS4 was completely inactivated after heat treatment at 60, 70, 90, 100, and 110 °C. Table 5 showed a comparison of ZOI among treated pediocin GS4 at different temperatures and duration against reference strains.

Effect of different organic solvents on the activity of pediocin GS4

The effect of different organic solvents such as methanol, ethanol, acetone, and hexane on pediocin GS4 was determined after 24 h of incubation at 37 °C by the agar well diffusion method. The treated pediocin GS4 was completely inactivated and did not show any antibacterial activity indicating its sensitivity towards laboratory solvents.

Discussion

Antagonism is an important and inherent property of being a good probiotic strain. The antagonistic property of *P. pentosaceus* GS4 has been demonstrated experimentally in competition with classical reference strains, and it thus adds value to the *P. pentosaceus* GS4 for beneficial application. It is seen that the *P. pentosaceus* GS4 can inhibit the growth of tested reference strains of Gram-positive and Gram-negative bacteria with clear demonstration of bactericidal activity. As *P. pentosaceus* GS4 is a homofermentative lactic acid producing, so the demonstrated antagonism may be shared due to the acid effect. But the neutralized CFS shows the similar result of inhibition approving the ability of GS4 for bacteriocin-like protein production. Millette et al. (2007) deduced bactericidal effect could be characterized as the production of organic acids in combination with the production of bacteriocin-like proteins which are active in acidic condition. It can be inferred that higher antimicrobial activity of fresh CFS of *P. pentosaceus* GS4 can be attributed to the synergistic effect of lactic acid and other antimicrobials such as ethanol (of heterofermentative LAB) and hydrogen peroxide as well as protein-like antimicrobials (bacteriocins). As we reported before, our strain GS4 has the ability of lactic acid (2.60 ± 0.01 g/20 mL of supernatant) production (Sukumar and Ghosh 2010a, b) and bacteriocin-like protein (this study) but does not produce ethanol and hydrogen peroxide (unpublished data). Barrow et al. (1980) pointed out that growth of most coliform bacteria could be inhibited when the pH of the medium was below 4.5. It was also observed that change in pH by 0.4 U was observed after 6 h for *L. gasseri* and 8 h for *P. pentosaceus* GS4 respectively (Barrow et al. 1980). Hence, it can be inferred that the *P. pentosaceus* GS4 and *L. gasseri* have slow acidifying potential (Fig. 2). Slow acidification property of the strain GS4 also thus demonstrates it being a good probiotic strain.

Gilliland and Speck (1977) reported that LAB strains showed stronger bactericidal properties against the Gram-positive bacteria than the Gram-negative bacteria by the elaboration of both organic acids and bacteriocin-like compounds. Silva et al. (1987) reported similar findings of the inhibitory activity of *L. rhamnosus* strain GG against members of *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* as well as anaerobic bacterium such as *Clostridium* through the production of low molecular weight antimicrobials. Vizoso Pinto et al. (2006) demonstrated the antagonistic activity of LAB towards several foodborne

	S. aureus	*L. monocytogenes*	*P. aeruginosa*	*E. coli*
Pediocin with 80 µg/100 mL				
α-Amylase (mg/mL)	20.0 ± 0.3	14.8 ± 0.4	16.8 ± 0.3	12.1 ± 0.1
Pepsin (mg/mL)	No ZOI			
Lysozyme (mg/mL)	15.4 ± 0.2	10.5 ± 0.3	No ZOI	12.4 ± 0.3

Table 3 Effect of enzyme-treated pediocin GS4 against reference bacterial strains (mean ZOI in mm)

Table 4 Effect of different pH-treated pediocin GS4 against reference bacterial strains (mean ZOI in mm)

pH	S. aureus	L. monocytogenes	P. aeruginosa	E. coli
3.0	No ZOI			
5.0	No ZOI	18.50 ± 0.6	22.00 ± 0.7	No ZOI
7.0	19.00 ± 0.4	27.00 ± 0.4	27.50 ± 0.8	17.50 ± 0.5
9.0	No ZOI			
12.0	No ZOI			

pathogens like *S. aureus*, *E. coli*, and *L. monocytogenes* due to production of organic acids and bacteriocin-like proteins.

In general, LAB are active against closely related species. In this study, *P. pentosaceus* GS4-nCFS showed broad spectrum antibacterial activity. Similar result was observed by Şimsek et al. (2006) and was reported the antibacterial activity against both Gram-positive and Gram-negative bacteria including *S. aureus*, *L. monocytogenes*, and *E. coli*. Such broad spectrum activity of *Lactobacilli* has been also reported by Atassi et al. (2006) where the inhibition of uropathogenic *E. coli* and the vaginosis associated bacteria such as *Gardnerella vaginalis* and *Prevotella bivia* was noted. The antimicrobial activity of the neutralized CFS infers the presence of bacteriocin-like antimicrobials. The antibacterial activity associated with the production of bacteriocin-like compounds can be exploited in the food industry as nutraceuticals.

The nCFS of *P. pentosaceus* GS4 showed inhibitory activity against *S. aureus*, *L. monocytogenes*, *P. aeruginosa*, and *E. coli* strains as demonstrated by the enumeration of viable bacteria. A minimum of 300–500 μL of nCFS of

P. pentosaceus GS4 was found effective and inhibited complete growth after 4–5 h of treatment (Fig. 3). These findings are consistent with the reports of Coconnier et al. (1992) where the spent culture supernatant of *Lactobacillus acidophilus* strain LB decreased the viability of *S. aureus*, *L. monocytogenes*, and *E. coli* as well as *S. typhimurium*, *Shigella flexneri*, *Klebsiella pneumoniae*, and *Enterobacter* spp. (Hernández et al. 2005).

Comparison of bactericidal activity caused by neutralized CSF of *P. pentosaceus* GS4 with that of untreated control reference strains revealed that the inhibition was due to some inhibitory substances like bacteriocin-like protein molecules. In a separate study, Arboleya et al. (2011) demonstrated the inability of the non-neutralized culture supernatants of *Bifidobacterium* isolate to inhibit the growth of *S. aureus*. However in this study, *P. pentosaceus* GS4 showed significant antimicrobial activity against *S. aureus*. The assay with neutralized CFS gave inhibitory zone with a lesser diameter compared to that of fresh. The fresh CFS possessed bacteriocin with lactic acid. Such CFS of *P. pentosaceus* GS4 gave the maximum ZOI against *S. aureus*, *P. aeruginosa*, *E. coli*, and *L. monocytogenes* with average zones of 17.53 ± 0.8 mm, 14.06 ± 0.5 mm, 13.20 ± 0.7 mm, and 13.73 ± 0.5 mm respectively (Table 1). Bacteriocin-like inhibitory compounds produced by *Enterococci faecium* inhibited a range of Gram-positive bacteria but not against the Gram-negative bacteria (Strompfová et al. 2004). However in the present study, *P. pentosaceus* GS4-nCFS was found to have broad spectrum activity towards both Gram-positive and Gram-negative bacteria with higher magnitude of antibacterial activity. Gram-negative bacteria are generally resistant to bacteriocins from

Table 5 Effect of different temperature-treated pediocin GS4 against reference bacterial strains (mean ZOI in mm)

Temperature °C	S. aureus	L. monocytogenes	P. aeruginosa	E. coli
	Treatment for 15 min			
30	20.00 ± 0.6	21.00 ± 0.9	34.00 ± 0.8	16.50 ± 0.3
40	20.50 ± 0.5	22.00 ± 0.5	33.50 ± 0.7	17.00 ± 0.5
50	20.50 ± 0.8	20.50 ± 0.9	33.00 ± 0.9	16.00 ± 0.9
60	No ZOI			
70	No ZOI			
90	No ZOI			
100	No ZOI			
110	No ZOI			
PBS (control)	No ZOI			
	Treatment for 30 min			
30	19.00 ± 0.3	22.00 ± 0.9	29.00 ± 0.6	13.50 ± 0.7
40	19.50 ± 0.8	23.00 ± 0.5	27.50 ± 0.9	14.50 ± 0.9
50	18.50 ± 0.7	21.50 ± 0.7	27.50 ± 0.9	11.50 ± 0.7
60	No ZOI			
70	No ZOI			
90	No ZOI			
100	No ZOI			
110	No ZOI			
PBS (control)	No ZOI			

lactic acid bacteria due to their outer membrane providing a barrier to permeability of these protein-like substances.

Numerable peptides or proteinaceous compounds having antimicrobial activity have been isolated from different fermented food (Kim et al. 1992). The precipitated and dialyzed nCFS of *P. pentosaceus* GS4 showed the maximum ZOI against the Gram-positive bacteria in comparison to bacteriocin-like compound produced by *Bacillus licheniformis* ZJU12 at the same percentage of saturation (45%) (He et al. 2006). The ZOI exhibited by isolated protein (pediocin GS4) against Gram-positive bacteria *S. aureus* and *L. monocytogenes* and as well as against Gram-negative bacteria *E. coli* and *P. aeruginosa* with higher ZOI than shown the bacteriocin produced from *Lactobacillus plantarum* strain isolated from marine shrimp (*Penaeus monodon*) gut (Karthikeyan and Santosh 2013). The measured ZOI by pediocin GS4 is 15.25 ± 1.2 mm against *L. monocytogenes* while in case of pediocin PA-1 it is reported up to 12.5 mm. Pediocin GS4 exhibited its activity against *E. coli* while in previously reported bacteriocin which was isolated from different sources, did not show any activity against *E. coli* (Deraz et al. 2005; Naghmouchi et al. 2011). The pediocin GS4 showed also antibacterial activity against *S. aureus* however bacteriocin isolated from *Lactobacillus acidophilus* IBB 801 did not show any activity against it, as reported elsewhere (Zamfir et al. 1999).

There have been found a great variance in molecular weight of the bacteriocin isolated from different sources, ranges approximately from 2700 to 16,599 Da (Bhunia et al. 1987; Gonzalez and Kunka 1987). The molecular weight shown by the pediocin F is 4460 Da as reported elsewhere (Osmanagaoglu et al. 1998a, b). The estimated molecular weight of pediocin GS4 is 9571 Da and is observed to be higher than the molecular weight of the bacteriocin produced from *L. plantarum* (Karthikeyan and Santosh 2013). The purity of the pediocin GS4 was examined by RP-HPLS and was found pure in comparison with nisin with the demonstration of a single peak (Fig. 5), however various peaks appeared from chromatographic analysis of pediocin AcM (Lopes et al. 2014). The secondary structure of pediocin GS4 was demonstrated by circular dichroism (CD) analysis revealing the possession of α-helix without β-sheet. This entails the stability of pediocin GS4 being active in broad range of pH and temperature (Lopes et al. 2014; Wei et al. 2014).

Bacteriocin-producing strains may play an important role in the maintenance of desirable population in the gastrointestinal tract by suppressing the growth of less desirable microbes (Pande et al. 2012; Ghosh 2013, 2018). Several other unique properties such as activity over a wide range of pH and temperature make pediocins suitable candidates as biopreservatives to extend the shelf life of food (Naghmouchi et al. 2011). There are several other reports of pediocin being partially purified and characterized (Bhunia et al. 1987;

Gonzalez and Kunka 1987). Thermostable pediocin NV 5 produced by *Pediococcus acidilactici* LAB 5 with bactericidal activity at low concentration has been partially purified (Mandal et al. 2008). Our study also demonstrates the bactericidal activity of pediocin GS4 at microliter concentration (low titer). This is the first report of antimicrobial potential of *P. pentosaceus* isolate from the Indian fermented food *Khadi*. In this direction, several *Lactobacilli* have been found to produce various types of antibiotic-like compounds or bacteriocins, like *L. acidophilus* produces lactacin while *L. plantarum* produces plantaricin (Andersson et al. 1988).

Pediocin is a class II-a type of heat-stable, membrane-acting antimicrobial peptide produced by various strains of *Pedicoccus* spp. The pediocin F produced by *Pediococcus acidilactici* F exhibited positive activity after treated it with the enzyme lysozyme against the reference bacterial strains of *Lactobacillus*, *Lactococcus*, *Leuconostocs*, *Pediococcus*, *Enterococcus*, and *Listeria* spp. other than *L. monocytogenes*, many of which are associated with spoilage of meat and dairy products as well as pathogenic bacteria (Osmanagaoglu et al. 1998a, b), whereas the purified pediocin GS4 exhibited its activity against not only *L. monocytogenes* but also showed activity against *S. aureus*, *P. aeruginosa*, and *E. coli*. The bacteriocin-producing *L. plantarum* strain isolated from marine shrimp (*P. monodon*) gut exhibited activity after treatment with amylase at concentration from 0.1 to 1 mg/mL whereas pediocin GS4 exhibited a large zone of inhibition after treatment with α-amylase at a concentration of 1 mg/mL (Karthikeyan and Santosh 2013). After treatment with the enzyme lysozyme at a final concentration of 75 mg/mL, the enterocin E-760 was not degraded and it exhibited the antibacterial activity whereas the lysozyme-treated pediocin GS4 also showed antibacterial activity at a final concentration of 1 mg/mL of lysozyme (Line et al. 2008). The bacteriocin produced by *Bacillus megaterium* 22 also showed the antibacterial activity only against *S. aureus* after treatment with pepsin and amylase at the concentration of 1 mg/mL and 2 mg/mL. The pediocin GS4 was not affected after treatment with α-amylase (1 mg/mL) as it exhibited good activity against *S. aureus*, *L. monocytogenes*, *E. coli*, and *P. aeruginosa* but it was completely degraded being treated with pepsin (1 mg/mL) and showed no activity against reference strains (Khalil and Elbahloul 2009). It thus proves pediocin GS4 is sensitive to pepsin (aspartate protease) and might have been cleaved to phenylalanine, tryptophan, and/or tyrosine respectively for which it lost its antibacterial property (Johnston et al. 2007). Pediocin GS4 showed better antibacterial activity against *S. aureus*, *L. monocytogenes*, *E. coli*, and *P. aeruginosa* after treatment with α-amylase and lysozymes in comparison with the previously reported bacteriocins (Drider et al. 2010; Todorov et al. 2010). Lysozyme-treated pediocin GS4 could possibly retain antibacterial property revealing the fact of absence of glycosidic bond in it (Venkataramani et al. 2013).

The bacteriocin L23 produced from *Lactobacillus fermentum* L23 exhibited its stability on treatment with pH 4.0 to 7.0 but it lost its activity when it was treated with pH 2.0, 3.0, 8.0, and 9.0 whereas pediocin GS4 exhibited its higher stability at pH 5.0 to 7.0 against reference bacterial strains (Pascual et al. 2008) and it corroborated with CD analysis. As reported elsewhere, enterocin S37, a bacteriocin incubated at different pH (4.0 to 9.0) for 2 h at 37 °C (Drider et al. 2010), a nisin-like bacteriocin produced by *Lactococcus* subspp., treated at pH 2.0 to 4.0 (Tuncer and Ozden 2010); enterocin E-760, treated for 2 and 24 h at 37 °C with pH ranges between 5.0 to 8.7 (Line et al. 2008), exhibited stability of antibacterial property. The antibacterial property was retained at pH 5.0 in a study where bacteriocin produced from *Lactobacillus* was incubated for 20 h at 30 °C, whereas pediocin GS4 showed the maximum activity after 1 h of incubation at 37 °C on treatment with pH between 5.0 and 7.0 (Karthikeyan and Santosh 2013). Several other unique properties such as activity over a wide range of pH and temperature may make pediocin GS4 a suitable candidate as biopreservative to extend the shelf life of food.

The bacteriocin produced from *Bacillus megaterium* 22 exhibited its resistance up to the temperature of 100 °C after 15 min of treatment in water bath where it was also treated at different temperatures such as 30, 40, 50, 60, 70, 80, 90, 100, and 121 °C. The bacteriocin was completely inactivated when the temperature was raised to 121 °C as it did not show any bacteriocin activity. Pediocin GS4 exhibited its higher activity on treatment with 30 °C for 15 and 30 min against *P. aeruginosa*. It can tolerate up to 50 °C after treatment for 15 and 30 min in water bath against other reference bacterial strains (Khalil and Elbahloul 2009). The bacteriocin produced from *L. plantarum* was incubated for 20 h at different temperatures from 10 to 60 °C where it exhibited the maximum and minimum activity after treatment at 40 °C and 30 °C respectively, whereas the pediocin GS4 showed the maximum antibacterial activity on treatment with 30 °C against *P. aeruginosa* and the minimum activity against *E. coli* after treatment with 50 °C for 15 and 30 min respectively (Karthikeyan and Santosh 2013). Piscicocin CS526 is a class II-a bacteriocin isolated from *Carnobacterium piscicola* CS526 exhibited its antibacterial activity after treatment at 100 °C for 15 min (Yamazaki et al. 2005). Likewise, enterocin E-760 exhibited its thermal stability at the concentration of 2 mg/mL with antibacterial activity when it was boiled for 5 min whereas pediocin GS4 exhibited its activity up to 50 °C of temperature with concentration of 0.8 mg/mL (Line et al. 2008). The pediocin GS4 indicated its purity by showing a single band in SDS-PAGE (9.57 kDa), a single peak (HPLC) with alpha helix configuration (CD). The antibacterial activity of pediocin GS4 was optimum at 50 °C and at pH 5.0 and 7.0.

With our previous reports on Probiotic *P. pentosaceus* GS4, the antimicrobial activity due to pediocin GS4 production will add much value to *P. pentosaceus* GS4 which can be used as prophylactic to help in restoration and maintenance of normal microflora of the intestine and to prevent enteric infection, prevent the side effects of antibiotic therapy, and many other immunomodulatory benefits to the host. The pediocin GS4, a type of bacteriocin, isolated from the *P. pentosaceus* GS4, appears to be a novel proteinaceous (9.57 kDa) inhibitor with broad spectrum antibacterial activity ensures its potential application in near future in biotechnology. The pediocin GS4 thus can be commercialized to produce different health care products like antibiotic creams or face washes as it possessed broad spectral bacteriocidal property and as food preservative.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals (if applicable) N/A

Informed consent N/A

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