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RESEARCH ARTICLE

Purification and characterisation of a novel bacteriocin produced by *Enterococcus faecalis* from the indigenous North East fermented food aakhone

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Abstract

Aakohone, a traditional Manipuri fermented dish made from sticky fermented soybeans, possesses unique clinically beneficial properties. Since these fermented foods have been consumed for generations, bacterial cultures isolated from them are unlikely to be perceived as foreign by the human immune system when used as probiotics. In this study, a bacteriocin-producing *Enterococcus faecalis* strain was isolated from aakhone. The culture-free supernatant was collected, precipitated and dialyzed. The antibacterial activity of the supernatant and dialyzed sample was tested against *Listeria monocytogenes*, *Escherichia coli* and *Streptococcus mutans*. Various physiological properties, including growth patterns, bacteriocin production and sensitivity to different enzymes, cholesterol reduction test, acid tolerance and biofilm activity were all studied, as well as the effects of temperature, pH and salinity. Using Lowry's method, the cell wall protein was isolated from the strain and quantified. Silver staining was used to identify the low molecular weight protein bands and to analyse their molecular weight. To purify the sample, column chromatography was used. After being lyophilized, the three fractions exhibiting antibacterial activity were applied to SDS-PAGE. According to the current study, *E. faecalis* from aakhone is a promising strain for future probiotic research.

Keywords: aakhone; bacteriocin; *E. faecalis;* fermented food

Introduction

Food is essential as it provides nutrients and energy for body growth, body functions, preventing diseases and it supports overall health. One of the earliest food preparation and preservation methods is the fermentation process used in ethnic cuisine. In addition to improving the food's physiochemical characteristics and nutritional value, it extends its shelf life. Fermented dishes made from soybeans are widely enjoyed around the world, but particularly in Asia. Some of the popular ethnic non-salted sticky fermented soybean snacks of the Eastern Himalayas are kinema, hawaijar, bekang, aakhone and peruyaan. During fermentation, microbial activity enhances the bioavailability of bioactive peptides, dietary fiber and isoflavones, contributing to their nutritional and health benefits (1). In Japan, miso, or fermented soybean paste, is a common culinary seasoning. Because of its beneficial physiological benefits, including its antioxidative activity, antimutagenic action and inhibitory effect on cholesterol formation, miso has been thought to contribute to the health and lifespan of Japanese people (2).

Lactic acid bacteria (LAB) are a frequent starter culture for dairy, meat and vegetable fermentations. Bacilli or cocci are aerobic, gram-positive and ferment carbohydrates to produce lactic acid. *Enterococci*, commonly known as LAB, can be found in feed, food and healthcare facilities in addition to the gastrointestinal systems of humans and animals. The results of a recent study use omics analysis to establish a connection between the ability of LAB biosynthesis and the antagonistic features of microbiome homeostasis, as well as their profiles in the human microbiome. Research into the mechanisms underlying LAB's protective effects for the host and microbiota should be prompted by the numerous and varied antagonistic SMs, which demonstrate the promise of LAB and their bacteriocins as alternative therapeutics (3, 8).

Most *Enterococcus faecalis* strains with the potential to promote health are included in the LAB and are referred to as probiotics. On the other hand, some strains specifically, vancomycin and multidrug-resistant enterococci (VRE) and MDRE are opportunistic pathogens that cause nosocomial infections and develop antibiotic resistance determinants that eventually result in intrinsic resistance to widely used antibiotics, thereby increasing the threat to global public health (4). Interestingly in a recent research on malignant HT-29 and AGS cell lines, the cytotoxic effects of *E. faecalis* secretions were comparable to those of the traditional anticancer medication Taxol (5). On normal cell lines, taxol is highly cytotoxic; however, on rapidly dividing FHs-74 normal cell lines, no significant cytotoxic effects were observed. Instead, the cell-free

supernatant from *E. faecalis* KUMS-T48 increased the expression of ErbB-2 and ErbB-3 genes, while taxol increased the expression of CASP-9, suggesting alternative apoptosis-inducing mechanisms of *E. faecalis* KUMS-T48. Additionally, the cell-free supernatant of *E. faecalis* KUMS-T48 exhibited notable antibacterial and anti-inflammatory properties. The apoptotic-induced cell-free supernatant's antibacterial or anti-proliferative activities were eliminated when being exposed to proteolytic enzymes. Therefore, these released proteins may be used as a drug to treat cancer. Naturally, prior to usage, a thorough analysis of their physicochemical, structural and functional characteristics should be conducted (5).

Bacteriocins, antimicrobial peptides produced by certain strains of *Enterococcus faecalis* were examined for 17 common bacteriocin-encoding genes of *Enterococcus* spp. in 65 *E. faecalis* isolates from 42 food samples and 22 clinical samples in Egypt. Eleven isolates (eight from clinical isolates and three from food), displayed that putative antibacterial activity. A combination of two peptides was produced by the food-isolated *E. faecalis* OS13 and bacteriocin-like inhibitory substances (BLIS) that prevented the growth of nosocomial *E. faecalis* and *E. faecium* isolates resistant to antibiotics (6).

Many strategies like feed additives, disinfectants and antibiotics are frequently used to control microbial growth. However, new approaches to treating bacterial infections are desperately needed because of their shortcomings-which include the emergence of resistance, low efficacy, high cost and negative impacts on food, health and the environment. Bacteriocins are promising antimicrobial agents due to their distinct mechanisms of action and diverse biochemical properties. It is emphasized how important bacteriocins are as next-generation antimicrobial agents and substitutes for antibiotics in various uses especially in food (7). For instance, many nations have employed the bacteriocin, nisin, which is generated by *Lactococcus lactis*, to preserve food (6).

Despite the effectiveness of antibiotics in inhibiting or eliminating infections, their misuse has led to the emergence of antibiotic resistance and even the development of "Super Bacteria." Thus, it is imperative to investigate safe, natural alternatives like bacteriocin (8). Lantibiotics, naturally occurring substances that are made by the ribosomes of Gram-positive bacteria, are members of the bacteriocins family of antimicrobial toxic peptides. There are two primary types of these antimicrobial peptides. Type B l antibiotics function by blocking vital enzymes and increasing binding to the target cell wall, whereas type A l antibiotics stop the bacterial cell wall from being produced or even destroy it by making holes in the cell wall. In addition, modified lantibiotics are being manufactured (8).

Recent studies have explored the use of rice bran for isolating LAB with antibacterial activity against *Bacillus subtilis*. One such isolate, *Enterococcus faecalis* RJ-11, showed a broad range of growth inhibition with different gram-positive bacteria. Enterocin RJ-11, a bacteriocin that was isolated from culture fluid, was sensitive to a number of proteolytic enzymes but heat stable and insensitive to acidic or alkaline environments. The examination of sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that enterocin RJ-11's monomeric form has a molecular weight of 5000. High degrees of similarity were found between the amino acid sequence of pure enterocin RJ-11 and the sequences of enterocins produced by *Enterococcus faecium* (2).

Similarly, *Enterococcus faecalis* DU10, a lactic acid bacteria isolated from fowl ducks, was purified using size exclusion chromatography and reverse-phase high-performance liquid chromatography with a C-18 column. It demonstrated a broad spectrum of antibacterial activity against significant food-borne pathogens against which it was tested. Using Tricine–SDS PAGE, a band with an estimated molecular mass of 6.3 kDa was found. The zymogram revealed a clear connection between this band and the antibacterial action. Mass-assisted laser desorption ionization time-of-flight mass spectrometry further supported this discovery by detecting a strong peak at 6.313 kDa and revealing the functional groups by Fourier transform infrared spectroscopy. Bacteriocin DU10 showed partial sensitivity to trypsin and α-chymotrypsin, but was susceptible to Proteinase-K and pepsin. Heat treatments ranging from 30 to 90 °C for 30 min only partially inhibited the action of bacteriocin DU10. Additionally, it endured a 10 min treatment at 121 °C. The methyl-thiazolyl-diphenyl-tetrazolium bromide test was used to measure the cytotoxicity of bacteriocin DU10 and the results indicated that the presence of 3200 AU/mL of bacteriocin reduced the viability of HT-29 and HeLa cells by 60 ± 0.7 % and 43 ± 4.8 %, respectively. The strain exhibited resistance to 0.3 % w/v bile oxygall and pH 2, which had an impact on bacterial growth after 2 to 4 hr of incubation. When adhesion characteristics were investigated using the HT-29 cell line, strain *E. faecalis* DU10 was shown to be highly attached to this substrate in 69.85 % of the initial population. The activity of bacteriocin DU10 was observed to be partially impacted by trypsin and α-chymotrypsin and susceptible to proteinase-K and pepsin. These findings suggest that *E. faecalis* DU10 may be utilized as a potential probiont to suppress Salmonella infections and that bacteriocin DU10 may be used as a potential bio preservative (9).This study focuses on the isolation and characterization of bacteriocins from aakohone (axone), a traditional alkaline fermented soybean food from Manipur, known for its medicinal properties.

The culture-free supernatant of *E. faecalis*, a bacteriocin -producing strain isolated from fermented food aakohone based on soy, was collected, precipitated and dialyzed. In the digestive tract, gram-positive lactic acid bacteria, like *E. faecalis*, are commonly observed. For the bacteriocin activity test, *L. monocytogenes*, *E. coli* and *S. mutans* were chosen as indicator organisms; the dialyzed material and supernatant were also utilized. Additionally, investigations were done in its growth, bacteriocin production, sensitivity to various enzymes, cholesterol reduction test, acid tolerance and biofilm activity.

Material and Methods

Bacterial strains and culture conditions

A bacteriocin producer strain *E. faecalis* A4 was previously isolated from the north east soya based fermented food aakhone in our laboratory by Sujith Kumar V. which was identified by morphological, physiological and biochemical tests and by a homology search based on the 16 rRNA sequence. The 16S rRNA sequence of *E. faecalis* A4 was

validated through a homology search using BLAST. The 16S rRNA sequence of *E. faecalis* A4 was validated through a homology search using BLAST. The partial sequencing of 16S rRNA gene sequence was performed by MACROGEN and sequence similarity search was carried out using BLASTn. Sequences of closely related taxa were retrieved and aligned using MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Identified probiotic strain is *Enterococcus facaelis* (provided as supplementary data).

Strain was maintained as frozen stocks at -80 ^{0}C at the Laboratory of Dr. V. Arul, Department of Biotechnology, Pondicherry University. MRS medium was routinely used for culturing E. *faecalis* A4.

Extraction of bacteriocin from probiont

Enterococcus faecalis A4 was inoculated in 200 mL of MRS broth and incubated for 16 hr at 37 ^{0}C. They were centrifuged at 8500 g for 20 min. The supernatant was collected and mixed with 75 % saturated ammonium sulphate and incubated for overnight at 40 °C. The precipitate was collected by centrifugation at 10000 g for 20 min at 40 °C. The precipitate was mixed with 10 mM phosphate buffer (pH 6) and dialyzed with phosphate buffer overnight using Spectra-pore dialysis tubing.

Well diffusion method to evaluate the antagonistic effect of supernatant isolated from probiotics over the pathogens grown in culture

The experiment was performed under strict aseptic conditions. After the medium solidified five-hour culture of serially diluted (10^{-3}) pathogenic bacteria was swabbed uniformly across the culture plate and well was made in the plates with sterile borer. The supernatant was introduced into the well and plates were incubated at 37 °C for overnight. The probiotic supernatant diffused radially from the well, inhibiting the growth of the pathogenic culture spread over the agar. All samples were tested in triplicates. The inhibition was determined by measuring the diameter of zone of inhibition around the well 6 hr culture of the probiotic strains were centrifuged at 6000 rpm for 6 min. 16 hr culture of pathogenic strains were serially diluted to 10^{-3}dilution and swabbed over BHI agar plates by using cotton swabs. Wells are prepared on agar plates by using well borer. 100 µl supernatant and pellet of the probiotic isolates were added separately inside the wells and the plates were incubated at 37 °C for 24 hr.

Characterization of bacteriocins

The crude bacteriocin samples were characterized with respect to thermal and pH stability, susceptibility to denaturation by enzymes, solvents and detergents. Further, the ability of the probiotic bacteria to withstand the effects of acidic conditions was also determined.

Thermal stability

Crude bacteriocin samples (1 mL each) were exposed to various heat treatments with temperatures 50, 60, 70, 80 and 90 °C for 10 min and 30 min. The residual anti-bacterial activity was then tested by the agar-well diffusion assay against the indicator organism *Listeria*. Untreated bacteriocin sample was used as a positive control.

pH Stability

Crude bacteriocin samples (3 mL each) were adjusted to pH 2, 4, 6, 8 and 10 with hydrochloric acid (HCl) and sodium hydroxide (NaOH) and incubated at room temperature for 4 hr. Anti-bacterial activity was then determined against the indicator organism by agar-well diffusion assay. Untreated bacteriocin sample was used as a positive control.

Sensitivity to solvents

One mL of crude bacteriocin sample was mixed with an equal volume of each of the given solvents (acetone, acetonitrile, ethanol, methanol, isopropanol and chloroform) and mixed thoroughly. The tubes were incubated at 37 °C for 1 hr and anti -bacterial activity was assessed against the indicator strains. Bacteriocin sample treated with 1 mL of distilled water was used as a positive control.

Sensitivity to detergents

One mL of crude bacteriocin sample was mixed with the following SDS, Tween- 20, Tween- 80 and Urea to obtain a final concentration of 1 % for each detergent. The tubes were incubated at 37 °C for 5 hr and anti-microbial activity was determined by the agar-well diffusion assay. Untreated bacteriocin sample was used as a positive control.

Sensitivity to enzymes

One mL of crude bacteriocin sample was mixed with 1 mL of each of the respective enzyme solutions and incubated at 37 °C for 2 hr. Bacteriocin treated with 1 mL of 50 mM sodium phosphate buffer was used a positive control. Following incubation, the samples were heated at 70 °C for 10 min to inactivate the enzymes. Residual anti-bacterial activity was analysed by the agar-well diffusion against the pathogenic strains.

Preparation of enzymes

Trypsin, chymotrypsin, lipase and catalase, all enzymes prepared for a final concentration of 1 mg/mL in 50 mM sodium phosphate buffer and Pepsin ++ (1mg/mL) in citric acid buffer.

Acid tolerance

100 µL of the probiotic strain under study was grown in MRS broth at 37 °C for 16 hr. The cells were harvested by centrifuging at 5000 x g for 10 min and the pellet was washed twice with 100 mM sodium phosphate buffer (pH 7.0). The cells were then suspended in the original volume with buffer by vortexing. 2 % cell suspension was inoculated in SGF and control SGF and incubated at 37 °C. Samples were collected at intervals of 0, 1, 2, 3, 4 and 24 hr to determine cell viability.

- Simulated Gastric Fluid (SGF) - prepared by dissolving 3.2 g/ L pepsin and 2.0 g/L sodium chloride (NaCl) into deionized water. The fluid was adjusted to the desired pH (i.e., pH 2.0-2.2) by addition of 1N HCl.

Control SGF - prepared as above but adjusted to a final pH of 6.5-7.0

Cholesterol reduction test

Freshly prepared MRS broth was supplemented with 0.3% bile salt. Water soluble cholesterol was filter sterilized and added to broth at a final concentration of 100 µg/mL 1 % of culture was inoculated in 20 mL MRS broth with an overnight culture of probiotic bacteria grown in MRS broth and incubate at 37 °C for 20 hr. Control was processed in the same way. Cells were removed by centrifugation at 8000xg for 5 min. Placed 0.5 mL of

supernatant into a clean glass tube. Added 1 mL of 33 % KOH followed by 2 mL of ethanol. Mixed after the addition of each component for 1 min. Heated the tubes in water bath at 37 °C for 15 min and cool to room temperature. Carefully added 3 mL hexane and 2 mL distilled water, mixed it vigorously in a vortex for 1 min. The tubes were allowed to settle at room temperature until complete phase separation occurs. Transferred 2 mL of the hexane layer (upper phase) into a clean dry test tube. Evaporated hexane to dryness at 60 °C under nitrogen gas flow. Resuspended the residue formed in 4 mL of o-phthalaldehyde reagent. Kept the tubes at room temperature for 10 min. Then pipetted 1 mL of conc. H_2SO_4 slowly down the inside of each tube. Mixed thoroughly and allowed to stand for 10 min at room temperature. Read the absorbance at 550 nm against the reagent blank. All the experiments were done in triplicates. The results are expressed as µg of cholesterol per mL.

- O-phthalaldehyde reagent - contains 0.5 mg of o-phthalaldehyhde per mL of glacial acetic acid (prepared fresh)

Anti-biofilm assay

Micro titer-plate test

For the micro titer-plate test, tryptic soy broth medium supplemented with glucose was used to grow the pathogens. Then the effect of the EPS (extracted from probiotic strain) on biofilm formation was investigated using a modified microtiter-plate method (10). The bacterial suspension (200 µl) was added to each of the three wells. The plate was covered with a lid and incubated anaerobically for 24 hr at 37 ^{0}C. Then the content of each well was aspirated. The wells were washed three times with 250 µl of sterile physiological phosphate buffered saline. The plate was shaken to remove non-adherent bacteria. The bacteria attached to the walls were then fixed with 200 µl of 99 % methanol per well and after 15 min the methanol was decanted and the well was dried. Two hundred µl of 1 % crystal violet were added to the wells to stain the biofilm cells for 5 min. The plate was placed under running tap water to rinse off the excess dye and then plates were air-dried. The dye bound to the adherent cells was re-dissolved with 200 µl glacial acetic acid per well. The optical density of the each well was measured at 570 nm using a plate reader (Triad multimode detector).

Mode of action

To determine the mode of action of bacteriocin 50 mg/mL of partial purified bacteriocin (freeze dried) was added to 4 hr-old cultures of *S. mutants* in 100 mL beef extract peptone medium (Final concentration was 0.5 mg protein/mL). The culture of *S. mutants* (without bacteriocin treated) were used as control. Changes in cell density were recorded at 600 nm.

Estimation of protein

Protein concentration was estimated following the method of Lowry (11). 0.1mL of crude extract was made up to 1 mL using distilled water. To this 5 mL of solution D was added and incubated at room temperature for 10 min. Then 0.5 mL of 1:3 diluted folin ciocallteau reagent was added and incubated in dark for 30 min. The absorbance was measured at 660 nm. Bovine serum albumin (1 mg/mL) was used as standard and distilled water is kept as blank.

Tricine SDS-PAGE

The gels of 1 mm thickness gel casting apparatus. The stacking gel contained 5 % acrylamide and the separating gel contained 15 % acrylamide The separating gel was poured between the sealed clean glass plates. Distilled water was layered on the top. After complete polymerization of the resolving gel, water on the top is blotted out and the stacking gel was poured onto the resolving gel up to the top. A suitable comb was inserted into the cassette and polymerization was allowed to take place. The gel cassette was then removed from the casting unit and placed on the electrophoresis platform carefully so that no air bubble is formed at the bottom of the gel in the lower tank. Then the comb was removed and upper tank is also filled with 1X buffer. Then the samples were loaded into the wells and run at 50 V.

Silver staining procedure

The polysaccharide gel was fixed in formaldehyde solution overnight and washed twice with distilled water. The gel was immersed into 50 % ethanol for 20 min and 100 mL of 0.01 % sodium thiosulphate solution was transferred into it. This was washed twice and treated with 0.2 % silver nitrate and kept in dark for 20 min. After washing, 100 mL of developing solution was added and rinsed until bands develop the reaction stopped using distilled water.

Purification of proteins by column chromatography Gel-Exclusion Chromatography

A glass column (1X20 cm) was packed with 25 g of sephadex G-25, which was swollen overnight in 150 mL of phosphate buffer (pH 6) containing 0.5 M NaCl. A two-bed volume of the buffer was used to stabilize the column. A reservoir was arranged in which the buffer level was maintained such that it is above the level of buffer in the column. The column was loaded with 2 mL of the dialyzed supernatant of PM8 culture. The flow rate was adjusted to 1 mL/min. The proteins were eluted using 10 mM phosphate buffer containing 0.5 M NaCl. Fifty-three fractions of 1.5 mL were collected during elution. All fractions were subjected to analysis of antagonistic activity. The fractions showing distinct peaks were pooled, lyophilized and stored at 4 ^{0}C for further use and checked for antimicrobial activity using *Listeria monocytogens* and *S. mutants* as indicator organisms. SDS page was also performed.

Results and Discussion

Antibacterial activity of isolated strains through well diffusion method

Antimicrobial spectrums of isolated strains were evaluated against several pathogens which includes *L. monocytogenes*, *E. coli* and *S. mutants*. The strain which demonstrated the best anti -bacterial activity was selected as indicator organism for further studies. Previous studies also showed fifteen enterocin-producing strains of *Enterococcus faecium* and *Enterococcus faecalis* were isolated from traditional Iranian lighvan and koozeh cheeses. The highest level of inhibitory activity were tested against the indicators of *Escherichia coli* and *Listeria innocua* (11). In our study the *E. coli* strains showed a zone inhibition of 14 mm on TSA plates (Fig. 1).

Extraction of bacteriocin from *E. faecalis* A4

Culture free supernatant of *E. faecalis* was collected and

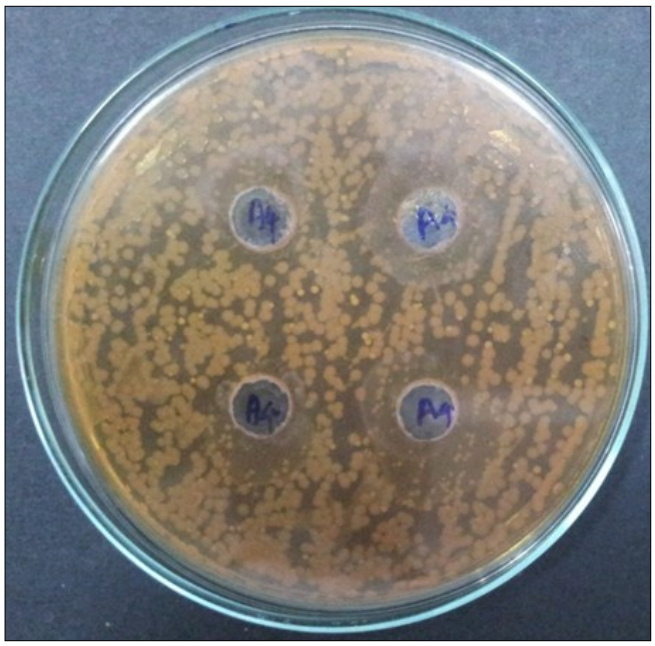

Fig. 1. Antagonistic effect of supernatant of isolated probiotics over *E. coli (*A4- supernatant of isolated probiotic bacteria).

precipitated by ammonium sulphate. The precipitated protein was dialyzed with phosphate buffer and then subjected to Tricine-PAGE electrophoresis along with protein molecular weight marker. The low molecular protein bands were observed and their molecular weight was analysed (12).

Characterisation of bacteriocin

We employed isolation and purification of *E. faecalis* A4, which yielded pure bacteriocin, small molecular weight enterocin that has the capacity to suppress both gram-positive and, in certain situations, gram-negative bacteria that are closely related (11). For its further use characterization of various parameters *invitro* are necessary.

Thermal stability

The effect of heat on the crude bacteriocin was investigated. Bacteriocin activity was maintained up to 60 °C for an exposure time of 15 min. However, when exposed for 30 min or longer, all activity was completely lost (Table 1). Several other studies have reported bacteriocins with varying degrees of susceptibility to heat. Bacteriocins from A4 have also been found to be heat-resistant and maintain anti-bacterial activity up to 60 °C for 10-15 min exposure time (Fig. 2). The bacteriocin can thus be said to

Table 1. Sensitivity of bacteriocin to temperature (Indicator organism - *L. monocytogenes*)

Temperature	Zone of inhibition (mm)	
	10 min	30 min
50 °C	11	15
60 °C	12	-
Control	11	15

possess moderate heat stability as previously reported (11). Temperature stability is important if the bacteriocins are to be used as a food preservative, because many procedures of food preparation involve a heating step.

pH stability

The pH stability of bacteriocin was assessed by adjusting its solution to different pH values (e.g., 2, 4, 6, 8, 10) using appropriate buffers, incubating at 37 °C for 1-2 hr, neutralizing the samples if necessary and then evaluating its antimicrobial activity through an agar well diffusion or microbroth dilution assay by measuring inhibition zones or bacterial growth reduction, where retained activity indicates pH stability while loss of activity at extreme pH suggests sensitivity. Bacteriocin A4 was found to be active at pH 2, 4, 6, 8, 10 and 12 (Table 2). However, maximum activity was observed around pH 10 and gradually decreased as pH was reduced to around 2 (Fig. 3). Activity was also retained in alkaline conditions at pH 4 and 8 and considerable activity was found at pH 10. This bacteriocin

Table 2. Bacteriocin activity at different pH

pH of bacteriocin	Zone of inhibition (mm)
2	25
4	13
6	10
8	12
Control	11

thus has the ability to remain active in both acidic and alkaline conditions as formerly testified (11).

Sensitivity to solvents

The fact that bacteriocins in different solvents still exhibit activity (Fig. 4) suggests that the bacteriocin in our investigation is not affected by the addition of solvents (Table 3). However, after prolonged exposure to organic solvents, bacteriocins are likely

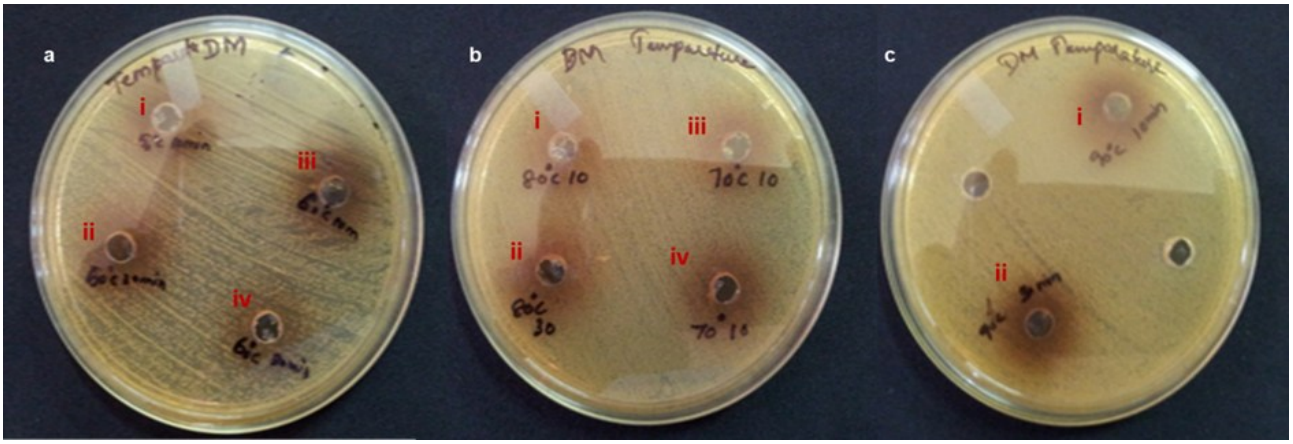

Fig. 2. Sensitivity of bacteriocin to temperature (Indicator organism - *L. monocytogenes*). a) Activity of bacteriocin at 50 °C& 60 °C at 10 & 30 min; b) Activity of bacteriocin at 70 °C & 80 °C at 10 & 30 min; c) Activity of bacteriocin at 90°C °C at 10 & 30 min.

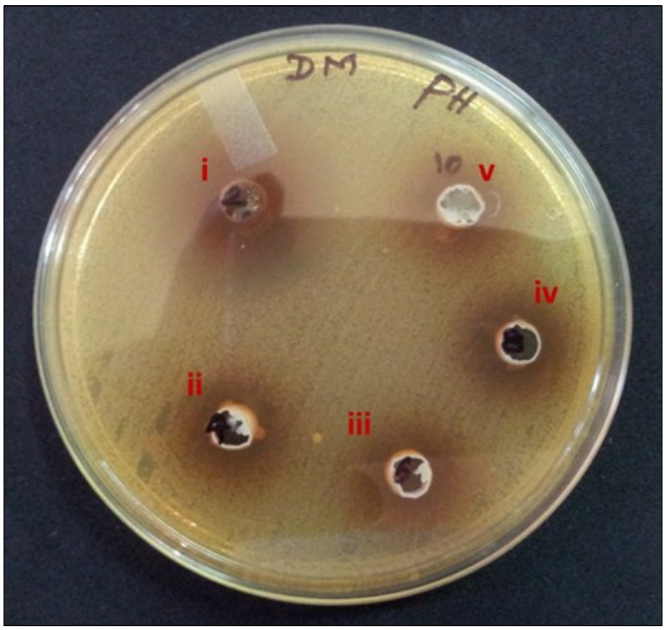

Fig. 3. Bacteriocin activity at different pH. Activity of bacteriocin at pH. i) 2; ii)4; iii) 6; iv) 8; v) 10.

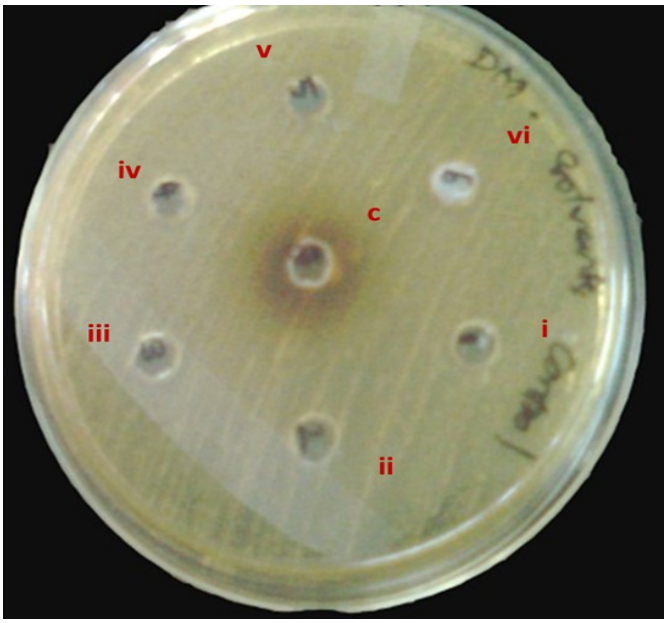

Fig. 4. Stability of bacteriocin to solvents. c) Control; i) Acetone; ii) Acetonitrile; iii) Ethanol; iv) Methanol; v) Chloroform.

Table 3. Stability of bacteriocin to solvents

Solvents used	Zone of inhibition
Acetone	10mm
Acetonitrile	10mm
Ethanol	Nil
Methanol	Nil
Isopropanol	12 mm
Chloroform	Nil
Control	12 mm

to misfold or clump, which will ultimately lead to their inactivation (13).

Sensitivity to detergents

The way detergents affect bacteriocin activity reveals details about the active molecule's structure. The hydrophobic core of proteins' native structures forms a compound with detergents, which causes the protein's three-dimensional structure to open and deteriorate. The reduction in activity after detergent treatment may be due to partial denaturation or loss of interactions with stabilizing molecules It has been shown that when proteolytic enzyme treatment is applied, protein-based bacteriocins fail to stable and lose their antibacterial efficacy. Enterocins have been shown by numerous researchers to be susceptible to one or more proteolytic enzymes.

Here the treatment of the bacteriocin with Tween-20, Tween-80, urea and SDS (Fig. 5)markedly increased the activity of the bacteriocin against *Listeria* (14). These findings (Table 4)

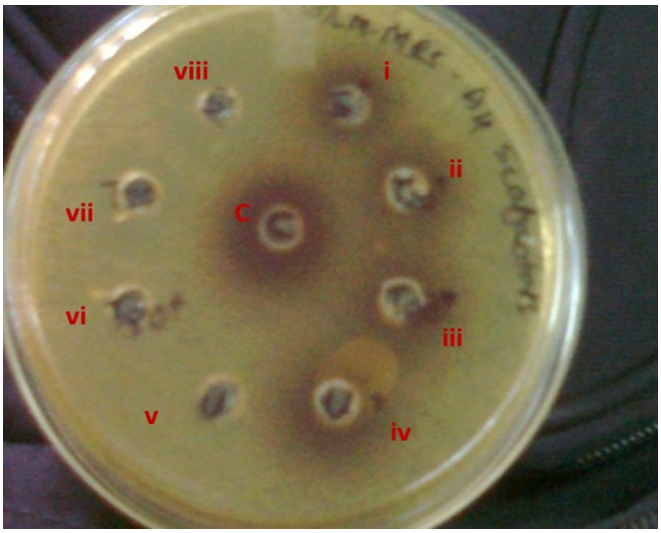

Fig. 5. Sensitivity to detergents.

Table 4. Sensitivity to detergents

Detergent used	Zone of inhibition	
	SAMPLE	CONTROL
Tween 20	14mm	14mm
Tween 80	10mm	-
SDS	13mm	17mm
Urea	-	-

are in line with the literature and verify that the bacteriocin has a proteinaceous structure (15).

Sensitivity to enzymes

Several reports have suggested that bacteriocins are proteinaceous in nature and thus exhibit loss of activity upon treatment with protein-hydrolyzing enzymes (16). Bacteriocin A4 retained activity after treatment with proteolytic enzymes such as pepsin, trypsin and catalase (Fig. 6). Thus, the bacteriocin can be said to be of proteinaceous nature and retains their activity upon treatment with enzymes like chymotrypsin (Table 5).

Acid tolerance

Bacteriocin A4 was viable at pH 2.0 even after 24 hr of incubation; however, the probiotic strain was lower compared to control (pH 6.0). Initially, when probiotic was inoculated, optical density (OD) at 600 nm. Then it was incubated at 37 °C after 2 hr interval the value was reduced gradually, after 24 hr. In other sense, there was reduction in bacterial absorption at pH 7.0. But it grows in pH 2.0 and pH 7.0 and majority of cells were viable at pH 2.0 even after 24 hr of incubation (Fig. 7) as already reported (11).

Cholesterol reduction test

The ability of the probiotic bacteria to assimilate cholesterol was studied according to the colorimetric method of an earlier study

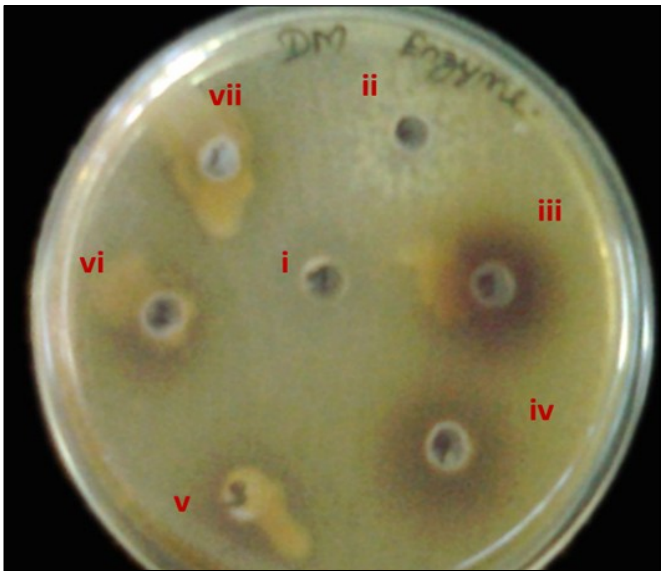

Fig. 6. Sensitivity of bacteriocin to enzymes. i) Pepsin; ii) Trypsin; iii) Chymotrypsin; iv) Catalase; v) Control; vi) Phosphate buffer; vii) Citric acid buffer.

Table 5. Sensitivity of bacteriocins to enzymes

Enzymes used	Zone of inhibition
Pepsin	**-**
Trypsin	**-**
Chymotrypsin	**12**
Catalase	**-**
+ve control	**14mm**
Citric acid buffer	**-**
Sodium Phosphate buffer	**10mm**

(17). The results were reported in terms of µg/mL of cholesterol assimilated by the organism. The amount of cholesterol remaining in the test sample was found to be 84.8 µg/mL from an initial concentration of 100 µg/mL. This indicates a reduction of 15.2 µg/mL or 15.20 % reduction rate of cholesterol assimilation by PES5. Several other reports also demonstrate the ability of probiotic organisms to reduce cholesterol with efficiencies ranging from 4 % to 35 % (18) .

Anti-biofilm assay

The production of biofilms by AMR bacteria is a significant global concern about antimicrobial resistance. Biofilm formation one of the protective layer of the pathogen was found to be decreased in the presence of bacteriocin. *Proteus mirabilis*, *Klebsiella pneumoniae*, *P. aeruginosa, L. monocytogenes*, *S. aureus*, *E. Faecalis, E. coli, Staphylococcus epidermidis, Staphylococcus viridans* and *E. coli* are a few examples of the gram-positive and Gram-negative bacteria that can produce biofilms. Similar investigations revealed that Enterocin MSW5 has eradicated S. aureus, *S. typhimurium* and *L. monocytogenes* biofilms with 53.51 ± 0.75 %, 30.02 ± 0.81 % and 42.18 ± 0.75 % of success, respectively (19). Consequently, we used a microtiter plate test to investigate the biofilm formation assay of three foodborne pathogens: *Listeria monocytogenes and S. mutants.*

In our study bacteriocin isolated from *E. Faecalis A* was showing good anti biofilm activity against pathogens like *Listeria monocytogenes and S. mutants.* Inhibition of biofilm formation by bacteriocin was optimum when bacteriocin concentration was 10 mg/mL (Fig. 8).

Mode of action of bacteriocin

The treated sample (*S. mutants* treated with bacteriocin) showed a decline in growth curve after 4 hr of incubation at 37 ^{0}C when compared with control (bacteriocin untreated *S. mutants*). Hence, observed as bacteriocin can resist the growth of the pathogen.

Estimation of protein

The cell wall protein was extracted from the strain *Enterococcus faecalis* and estimated by Lowry's method and found to have a bacteriocin concentration of 9.7 mg/mL.

SDS page

The low molecular protein bands were observed and their molecular weight was analyzed (6) (Fig. 9).

Purification of bacteriocin using column chromatography

The dialyzed protein sample was purified by gel filtration chromatography and Sephadex G-100. Fifty-three fractions were collected (Fig. 10) and their antimicrobial activity was tested against the indicator strain *S. mutans and L. monocytogenes*. Based on their activity, three fractions were selected and freeze

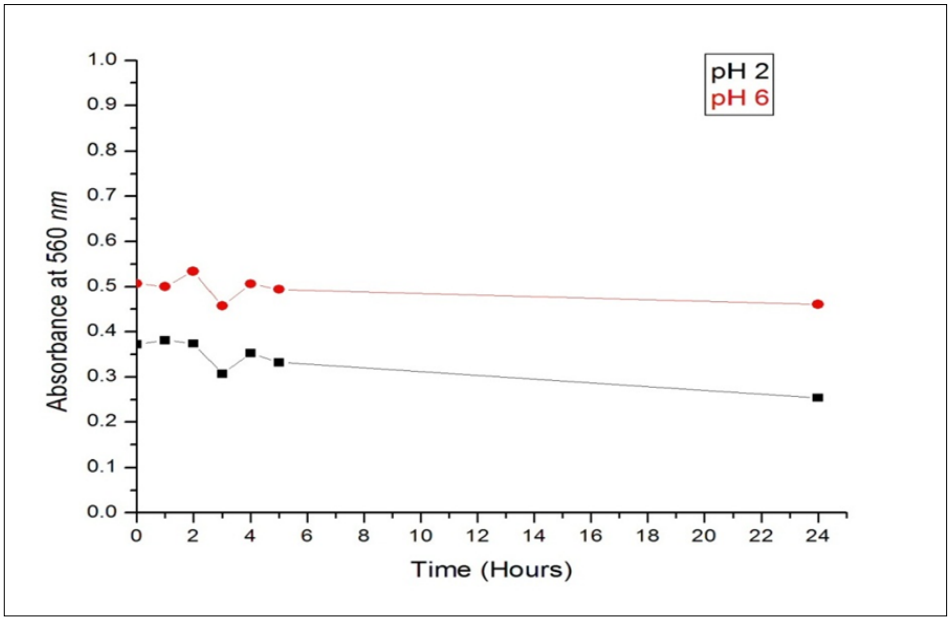

Fig. 7. Acid tolerance of bacteriocin.

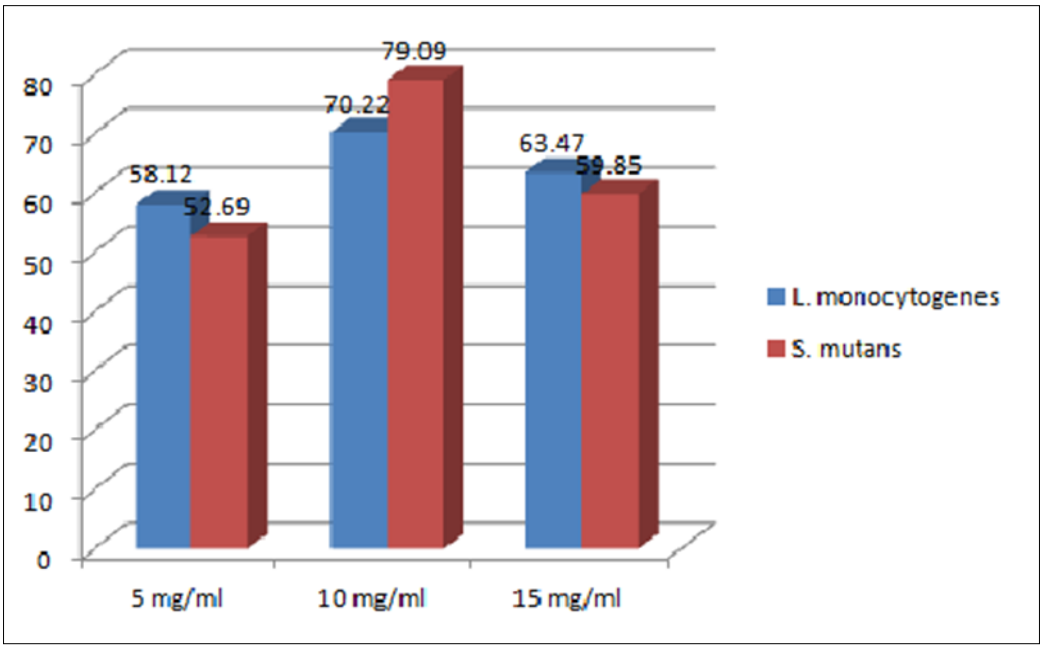

Fig. 8. Antibiofilm activity.

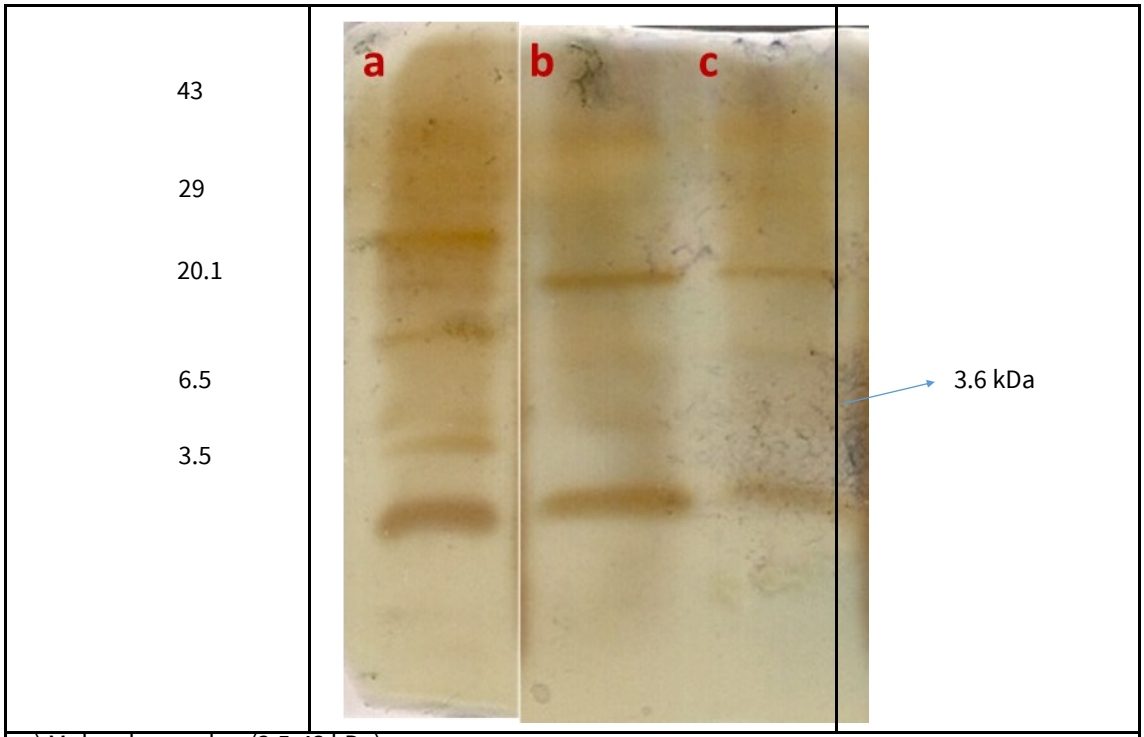

Fig. 9. Tricine SDS-PAGE of bacteriocin.

dried for further purification. SDS-PAGE was performed for the eight fractions that shown activity. The fractions 29 and 33 showed maximum antimicrobial activity (Fig. 11).

The maximum bioactivity of the collected part was found in fractions 17, 18 and 19. When purifying the bacteriocin via gel filtration, a single peak of protein (Fig. 10) was seen (20). Furthermore, it can be seen from results of *E. faecalis* bacteriocin fractions molecular weight determination that the purified bacteriocin's estimated molecular weight is 3.6 kDa (Fig. 12). The variations in bacterial strains and molecular weight determination techniques may be the cause of the molecular weight discrepancies. *E. faecalis* bacteriocin has been shown in multiple investigations to belong to type III bacteriocins, which are distinguished by large, heat-labile antimicrobial proteins (21) .

This study highlights a novel bacteriocin produced by *E. faecalis* isolated from the indigenous Northeast fermented food aakhone, which could serve as a functional probiotic bacterium. Locally sourced probiotic from traditional fermented food could be a valuable, healthful and reasonably priced natural therapeutic for the sustainable health of the local people. Biopreservatives, one of the primary products of microbial activity during fermentation, may nonetheless create special

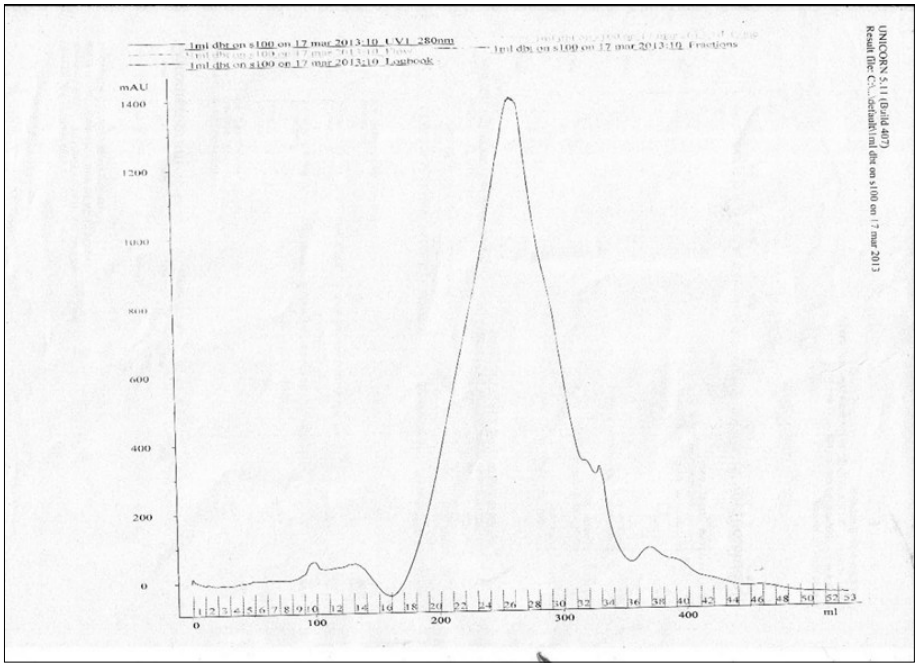

Fig. 10. Protein purification by gel exclusion chromatography.

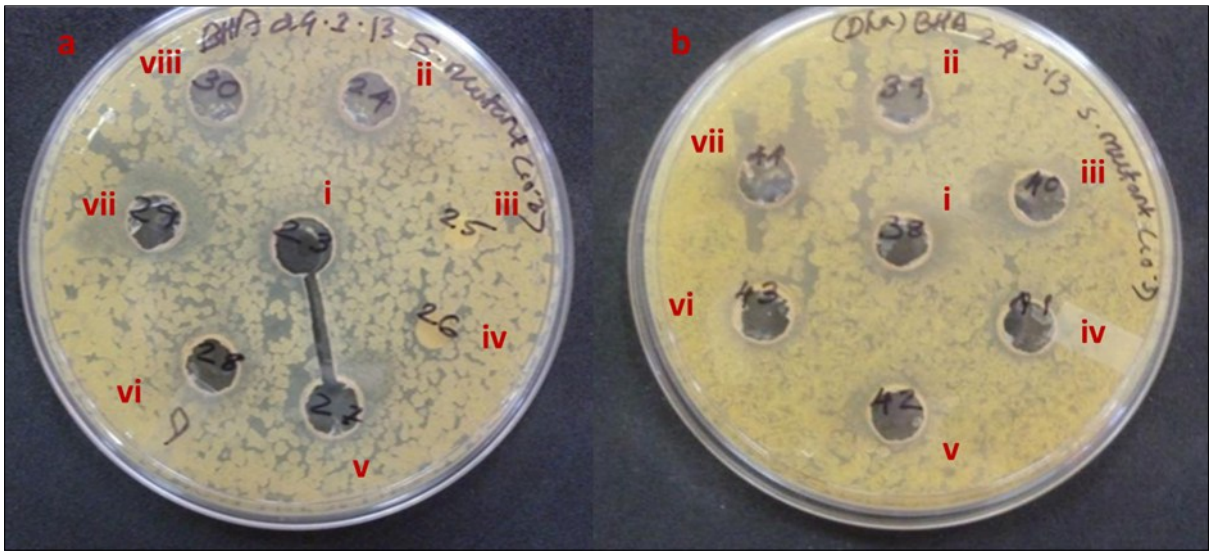

Fig. 11. Activity of partially purified bacteriocin factions against *S.mutans.*

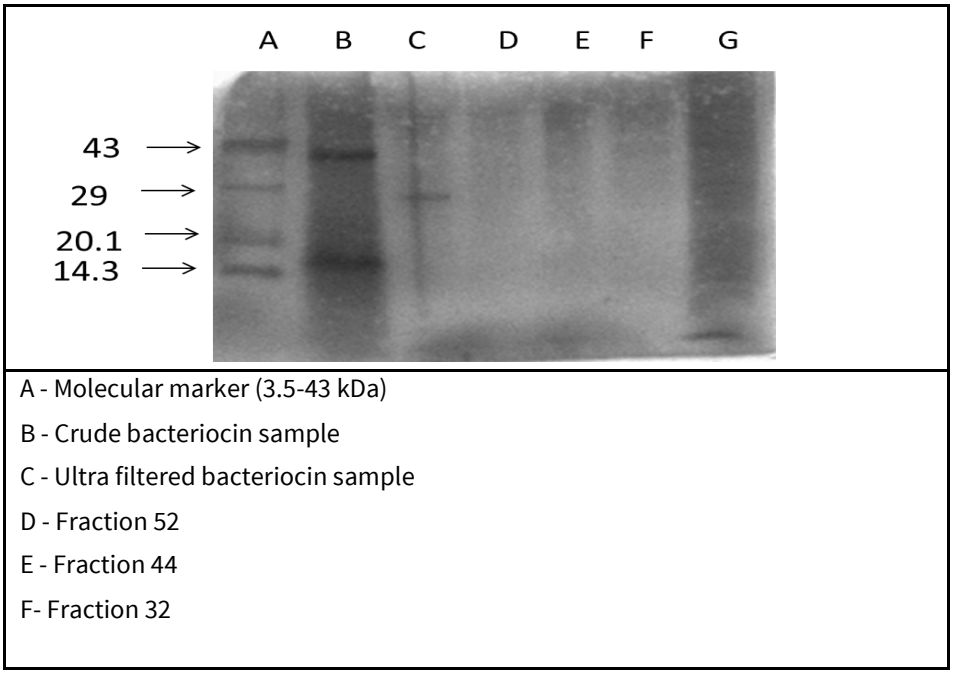

Fig. 12. SDS-PAGE for partially purified bacteriocin fractions.

substances that inhibit or eradicate pathogens includes lactic acid, acetic acid, propionic acid, H_2O_2, CO_2, ethanol, butanol, bacteriocin and other related chemicals.

In this study, we investigated the antimicrobial properties of bacteriocin against a range of pathogenic bacteria which includes *L. monocytogenes*, *E. coli* and *S. mutants*. The isolate with the best cumulative probiotic potential and reasonable persistence of supernatant and dialyzed E. *faecalis* A4 sample were used for the bacteriocin activity test with common food borne pathogens *L. monocytogenes*, *E. coli* and *S. mutans* selected indicating it as an effective biopreservative candidate.

In order to determine consumer safety, the probiotic, technical and safety aspects of these strains were assessed for optimum conditions for its growth, bacteriocin production, sensitivity to different enzymes , cholesterol reduction test, acid tolerance and biofilm activity were all studied, as well as the effects of temperature, pH and salinity. As previously reported, it was discovered to be stable across a broad pH and temperature range (19). The cell wall protein was isolated and quantified using Lowry's method to identify and analyze low molecular weight protein bands. To purify the sample, column chromatography was used. After being lyophilized, the three fractions exhibiting antibacterial activity were applied to SDS-PAGE. According to the current study, *E. faecalis* from aakhone is a promising strain for future probiotic research.

The identification and characterization of microorganisms present in traditional fermented goods should be given priority in future research on the microbial diversity in Indian food and beverages. Moreover, their implications for nutrition and food safety should be assessed. The possibility of using microbes to make fermented food and novel food products should be investigated further. It would also be advantageous to investigate techniques to increase the nutritional value of food and to better store and preserve it. Examining the health benefits of eating and drinking fermented food and drink, as well as how these benefits might vary by demographic and location, is one possible field of research. Thus, it is imperative to look into the potential effects of changes in food production, processing and distribution methods on the variety and safety of fermented foods and beverages in India.

Conclusion

E. faecalis A4 culture-free supernatant was collected, precipitated and dialyzed then were tested for bacteriocin activity against different infections using indicator strains, *L. monocytogenes*, *E. coli* and *S. mutans*. The study investigated the effects of salinity, pH, enzyme sensitivity, acid tolerance, cholesterol reduction, biofilm activity and temperature on bacteriocin production and bacterial growth. Using column chromatography, the dialyzed sample was purified. The three fractions that exhibited antibacterial activity were lyophilized and utilized for the SDS-PAGE for further studies. According to the current study, *E. faecalis* from soya based North East fermented food aakhone is a promising strain for future probiotic research. More studies to access toxicity, stability and *in vivo* testing are needed for its safe development as a promising probiotic strain.

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Authors' contributions

DM carried out the studies and drafted the manuscript. VP conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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