Characterization and Cytotoxic Evaluation of Bacteriocins Possessing Antibiofilm Activity Produced by *Lactobacillus plantarum* SJ33

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Abstract

Biofilm forming pathogens are among the major causes of hospital-acquired infections and are not much affected by antibiotic treatment. Consequently, novel agents and therapeutics are required urgently that possess antibacterial and antibiofilm activities. This study analyzed two bacteriocins from *Lactobacillus plantarum* subsp. *argentoratensis* SJ33 strain for their antibacterial and antibiofilm activity as well as cytotoxic properties. BacF1 and BacF2 showed broad spectrum activity against both Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*) bacteria. Significant bactericidal action was also observed on *S. aureus* cells by pore formation. Additionally, bacteriocins disrupted biofilms formed by *S. aureus* and *P. aeruginosa* which were shown by crystal violet staining assay and visualized by fluorescence as well as scanning electron microscopy. Quantitative Real-Time PCR study revealed changes in gene expression of biofilm formation in *S. aureus* (*ica*) and *P. aeruginosa* (*pelA*, *psl*, *rhlA*). Cytotoxicity of bacteriocins was further analyzed on normal mammalian cells and *Caenorhabditis elegans*. Notably, bacteriocins showed no major effect on HEK-293 cell line and enhanced the survival of *S. aureus* infected HEK-293 cells. Similarly, no cytotoxic effect was visible on *C. elegans* even after treatment with higher concentration than MIC at different time intervals.

Keywords *Lactobacillus plantarum* SJ33 · Bacteriocins · *S. aureus* · Antibiofilm · Quantitative real-time PCR · Cytotoxicity

Introduction

Biofilms are microbial communities embedded in a self-secreting extracellular matrix attached on an abiotic or biotic surface. Organisms inside the biofilms can escape the external environment and limit the entry of antimicrobial agents thus developing more resistance towards antibiotics (Tan et al. 2018). Bacterial biofilms are mainly composed of extracellular polymeric substances (EPS) such as polysaccharides, proteins, metabolites and extracellular DNA (Okuda et al. 2013). Most of bacterial infections are caused by biofilms which are formed by various pathogens and

are reasons of serious concern to human health. Bacterial adhesion and formation of biofilms can amplify the risk of biofilm-associated infections. According to the present data, biofilm plays a vital role in nearly 80% of human infections (Cirkovic et al. 2016).

Staphylococcus includes Gram-positive pathogens, specifically *Staphylococcus aureus*, an opportunistic pathogen which forms biofilms by secreting exopolysaccharides and proteins. Polysaccharide intercellular adhesin (PIA), the major component of *S. aureus* biofilms is regulated by *icaADBC* operon (Xu et al. 2017). *S. aureus* is one of the major causes of community-acquired and nosocomial infections. *Pseudomonas aeruginosa*, Gram-negative bacteria are another important opportunistic pathogen that causes several infections in clinical settings and are among the common causes of hospital-acquired and medical device-related infections (Gerits et al. 2016). Conventional antibiotics that are active against planktonic bacteria are often ineffective against biofilms formed by pathogens (Vahedi Shahandashti et al. 2016). Hence, biofilm-associated infections have

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become a major healthcare challenge in the current times and has urged for the requirement of alternatives and new therapies to develop potent antibiofilm agents (Konai and Haldar 2016).

Bacteriocins are small peptides and proteinaceous substances possessing antibacterial activity against various pathogenic microorganisms. Bacteriocins can be considered as alternatives to traditional antibiotics with already existing antimicrobial compounds either alone or in combination to target biofilms (Mathur et al. 2018). *Lactobacillus* species belongs to lactic acid bacteria (LAB) group that constitutes a major part of the gut microbiota. LABstrains are known to produce numerous bacteriocins and antimicrobial peptides that interfere with infections by inhibiting adherence of pathogenic bacteria (Ahn et al. 2018). Nisin is the most studied bacteriocin of LAB which was approved by FDA (Food and Drug Administration) and produced industrially but could mostly inhibit Gram-positive pathogens (Miao et al. 2014). Hence, the search for other bacteriocins with a wide antibacterial spectrum and better stability at broader temperature and pH range has been in constant focus and is gaining more attention (Zhang et al. 2018; Ibrahim et al. 2019). Different procedures have been used to analyze bacteriocins and the most common method being in vitro assays. It is difficult to perform in vivo cytotoxicity assays for bacteriocins involving direct mammalian models. So, to overcome these limitations, a simple in vivo screening method using *Caenorhabditis elegans* animal model was developed. *C. elegans* is a small nematode that feeds on *E. coli* and has been extensively used as an experimental model in biological studies due to its simplicity and appropriateness for genetic analysis (Son et al. 2016). Though *C. elegans* is one of the common models in laboratory setups, only few researches have focused on monitoring the effect of bacteriocins on growth of the worms under natural conditions.

Previously, we have shown the production of two low molecular weight bacteriocins by *Lactobacillus plantarum* subsp. *argentoratensis* SJ33 strain in our earlier study (Ray Mohapatra and Jeevaratnam 2019) and demonstrated its antibacterial and antibiofilm activities as well as therapeutic potential of bacteriocins as antibacterial coating on urinary catheters, thereby reducing the occurrence of medical device-associated infections (Ray Mohapatra and Jeevaratnam 2019). The present study focused to further characterize and analyze the antibacterial potential of individually purified bacteriocins, BacF1 and BacF2 on planktonic as well as biofilm forming pathogens. The study also evaluates the cytotoxic effect of bacteriocins on HEK-293 cells and *C. elegans*.

Materials and Methods

Bacterial Strains and Growth Media

All pathogenic bacterial strains used in the study (Table 1) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTech), Chandigarh, India. The pathogenic strains were grown using Tryptic Soya broth (TSB) and agar media (TSA) (HiMedia, India). *Lactobacillus plantarum* subsp. *argentoratensis* SJ33 was grown and maintained in Man Rogosa Sharpe (MRS) medium. Pure bacterial cultures were maintained with 50% glycerol and stored at −80 °C.

Growth and Maintenance of *Caenorhabditis elegans* Strain

The wild type N2 *C. elegans* and the bacteria *E. coli* OP50 was procured from Caenorhabditis Genetics Center, USA. The wild type strains of *C. elegans* were grown and maintained at 20 °C in Petri plates containing Nematode Growth Medium (NGM) and were cultivated with alive *E. coli* OP50 (Gusarov et al. 2013). The eggs were acquired from the grown adult worms after being treated with M9 buffer containing NaOCl -KOH solution. After hatching, the L1 stage worms were transferred onto the NGM agar plates containing *E. coli* OP50 and incubated on agar plates till it obtains the L4 stage (Fabian and Johnson 1994).

Table 1 Antibacterial spectrum of purified bacteriocins BacF1 and BacF2 from SJ33

Indicator strain	Zone of inhibition	
	BacF1	BacF2
Listeria monocytogenes MTCC 657	20 ± 0.57	18 ± 1.15
Staphylococcus aureus MTCC 737	17 ± 0.57	15 ± 0.57
Staphylococcus aureus MTCC 96	18 ± 1.15	16 ± 1.15
Klebsiella pneumoniae MTCC 109	16 ± 1.15	13 ± 0.57
Klebsiella pneumoniae MTCC 3384	16 ± 1.15	14 ± 0.57
Aeromonas hydrophila MTCC 1739	17 ± 0.57	16 ± 1.15
Pseudomonas aeruginosa MTCC 2295	16 ± 1.15	14 ± 0.57
Pseudomonas aeruginosa MTCC 3541	15 ± 0.57	13 ± 0.57
Escherichia coli MTCC 728	17 ± 0.57	14 ± 1.15
Bacillus subtilis MTCC 619	16 ± 0.57	15 ± 1.15

Inhibition zone in millimeters (mm) including well diameter of 6 mm; values are expressed in mean ± SE of three independent experiments in triplicates

Purification and Characterization of Bacteriocins

The partially purified bacteriocin from *Lactobacillus plantarum* SJ33 obtained in our previous study (Ray Mohapatra and Jeevaratnam 2019) was further purified by semi-preparative RP-HPLC and subjected to analytical RP-HPLC for analyzing the purity of bacteriocins.

Molecular mass of purified bacteriocins was identified by discontinuous Tris-tricine SDS-PAGE method (Hermann Schägger 2006). The electrophoresis was performed by using molecular weight marker of ultralow range from 1.06 to 26.6 kDa (Sigma-Aldrich, India) as standard with the purified bacteriocins. The gel was stained with Coomassie Brilliant Blue G-250 dye for 2–4 h and destained to visualize the bands. The molecular mass of bacteriocins was confirmed in our previous study by Q-TOF (Q-TOF SYNAPT G2) ESI mass spectrometer (Ray Mohapatra and Jeevaratnam 2019).

Antibacterial Activity

Bacteriocin activity was tested by agar well diffusion assay after serial dilutions (Hernández et al. 2005). An overnight culture of indicator strains (Table 1) was grown in TSB medium and seeded by pour plate method and purified bacteriocins were placed on solidified agar in 6 mm diameter wells and the plates were incubated at 37 °C for 24 h. Spectrum of bacteriocins activity was determined against various Gram-positive and Gram-negative pathogens (Table 1) by well diffusion method.

Effect of Protease Enzyme on Bacteriocins

Efficacy of protease enzyme on bacteriocins activity was determined by treating purified bacteriocins with 1 mg/mL of protease enzyme. Bacteriocins treated with protease enzyme (1 mg/mL) were incubated at 37 °C for overnight 10 mM citrate buffer (pH 3.0). Bacteriocins fractions were then adjusted to pH 6.0 and the antibacterial activity was tested against *S. aureus* MTCC 96 by agar well diffusion assay (Lin and Pan 2019) and compared with control which was processed by same method but not treated with the enzyme (Agaliya and Jeevaratnam 2013).

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of purified bacteriocins was tested against *S. aureus* MTCC 96 using broth dilution assay. Different concentrations of bacteriocins (3.9 to 250 μM), 100 μL per well was added to the microtiter plate and Nisin was taken as standard for comparison. The indicator strain (10^6 CFU/mL) was instilled in each well and incubated at 37 °C for 24 h. The MIC values were considered as the lowermost concentration at which the bacteriocins visibly inhibited the bacterial growth. Broth media with bacterial culture was taken as negative control whereas medium containing only bacteriocins without indicator strain was considered as positive control; both the controls were maintained with the test samples.

Time-Kill Assay

Overnight culture of *S. aureus* MTCC 96 (10^6 CFU/mL) was diluted (1:100) with TSB media and incubated for 2 h at 37 ℃ was used for growth kinetic assay. After incubation, the bacteriocins (MIC) were added to the indicator strain. Both untreated and treated cultures were incubated up to 12 h at 37 ℃ (Sahoo et al. 2015). The viable cell count of pathogen was measured after appropriate dilutions of both untreated and treated bacteriocins (MIC), then these cultures were used for plating on agar medium at 4th, 8th and 12th h (Kindoli et al. 2012). The number of colonies formed after bacteriocin treatment was determined and compared with untreated bacterial cultures after incubation at 37 °C for 24 h. *S. aureus* MTCC 96 culture without any treatment of bacteriocins was used as control (Amortegui et al. 2014).

Scanning Electron Microscopy

Culture of *S. aureus* MTCC 96 was grown overnight, diluted (1:100) with the growth medium and added with bacteriocins at MIC. The samples were then incubated for 6 h to view under microscope for morphological changes as compared to untreated cells. The untreated and treated cells were thoroughly washed twice with PBS and fixed by 2.5% glutaraldehyde. After fixation, cells were further treated with 1% osmium tetroxide and finally dehydrated with a gradient series of ethanol (25, 50, 75, 90, 95 and 100%). The dehydrated cells were coated with gold and examined under HR SEM(FEI Quanta FEG 200, USA) for analysis of cell morphology.

Biofilm Assays

Congo Red Assay

Congo red stain with agar medium was prepared with 37 g/L brain heart infusion (BHI) broth, 50 g/L sucrose, 10 g/L agar and 8 g/L Congo red dye was added with 5% sucrose. Congo red stain was sterilized at 121 °C for 15 min independently from the other medium constituents. Then it was supplemented to the sterilized BHI agar with sucrose at 55 °C. Congo red agar (CRA) plates were inoculated with indicator organisms and incubated for 24 h at 37 °C aerobically (Mathur et al. 2006; Hassan et al. 2011).

Antibiofilm Activity

Biofilm formation by *S. aureus* MTCC 96 and *Pseudomonas aeruginosa* MTCC 3541 was determined qualitatively and quantitatively by crystal violet (CV) assay. Bacteriocins effect was examined on the biofilm forming pathogens with different concentrations and the microtitre plate was incubated for 24 h at 37 °C. After incubation, the unadhered bacteria were gently washed with Phosphate Buffer Saline (PBS) and the biofilms were stained with 0.1% crystal violet stain for 10 min. Wells with excess stain was gently rinsed with PBS and the attached stain was dissolved with 33% acetic acid (Overhage et al. 2008). The optical density was measured in microtitre plate reader at 590 nm. The biofilms of indicator strains without bacteriocins were considered as untreated control.

Visualization of Biofim Formation and Inhibition under Microscope

Biofilm formation and inhibition was observed under microscope by using 12-well polystyrene plates containing cover slips. The cover slips were treated with and without bacteriocins at sub-MIC (31.25 µM), which were placed in the 12-well plate containing *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 3541 cultures (1:100 dilution with TSB) with $OD_{600nm} \sim 0.01$ and incubated for 24 h at 37 ℃ (Wu et al. 2016). The cover slips were rinsed with PBS and stained with 0.01% of acridine orange (w/v) for 5 min. After staining, the cover slips were again rinsed with PBS to remove additional stain and finally viewed under fluorescence microscope (Olympus, Japan).

The inhibition of biofilm of *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 3541 by bacteriocins was also viewed under SEM. Biofilms formed by both the pathogens were developed on cover slips and the cover slips were added with bacteriocins at sub-MIC (31.25 µM) and incubated at 37 ℃ for 24 h. The cover slips were rinsed, fixed by 2.5% glutaraldehyde and then dehydrated with a gradient ethanol series (25–100%). The dehydrated cover slips were then gold coated for observation under HR SEM (FEI Quanta FEG 200, USA).

Quantitative Real-Time PCR

RNA was isolated from both bacteriocins treated and untreated biofilms and the RNA isolation was carried out using HiPurA Total RNA isolation kit (HiMedia, India). Dnase treatment was given to remove genomic DNA contamination. The quantity and quality of RNA were assessed by nanodrop and agarose gel electrophoresis analysis. One microgram of RNA was used to prepare cDNA by Reverse Transcriptase kit (Genei, Bangalore, India). Comparative

differential gene expression of *icaC* and *icaD* in *S. aureus* MTCC 96 as well as *pelA*, *psl*, *rhlA* genes in *P. aeruginosa* MTCC 3541 were analyzed by quantitative reverse transcription PCR (Lakshmanan et al. 2019). The primer sequences used in this analysis are mentioned in the Supplementary material Table S2. Reactions were carried out using KAPA SYBR master mix (Sigma, India) and the reaction condition includes, an initial denaturation at 95 °C for 2 min, then 40 cycles of three step amplification of denaturation at 95 °C for 3 min, annealing at 55 °C for 30 s and extension at 72 °C for 30 s run in Roche Light Cycler 96. The results were analyzed by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The gene expression levels were standardized against *gyrA* and *rpoD* constitutive genes in *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 3541, respectively.

Cytotoxicity Assays

Effect of Bacteriocins on Cell Viability

Human embryonic kidney (HEK-293) cell line was obtained from the NCCS, Pune and was grown and maintained in 90% Dulbecco Modified Eagle Medium (DMEM) containing HEPES buffer and 10% FBS with 0.1% of Penicillin and Streptomycin. The effect of bacteriocins on HEK-293 cell viability was analyzed using MTT assay (Sakurazawa and Ohkusa 2005). Two-fold serial dilutions of bacteriocins (ranging from 15.6 to 500 µM) were prepared with culture media which was added to the cells across the microtiter plate and kept in 5% CO_2 incubator at 37 °C for 24 h. The plate supplemented with MTT (0.25 mg/mL) was incubated for 4 h, 200 µL DMSO was added and the absorbance was finally recorded at 570 nm. Results were expressed as percentage of viable cells.

Cell Adherence Assay

HEK-293 cells at density of 10^4 cells were seeded and added to each well of microtitre plate for 24 h. The cells were treated with bacteriocins at MIC and sub-MIC (31.25 µM) whereas untreated control wells did not receive any bacteriocin treatment prior to infection with the pathogen. After incubation, overnight culture of *S. aureus* MTCC 96 (OD-0.01) was used to infect HEK-293 cell line and then cells were incubated for 4 h at 37 °C. After incubation, the cells were rinsed with PBS and gentamicin (100 µg/mL) was added to remove unattached bacteria. The cells were again rinsed with PBS and processed for MTT assay.

For fluorescence microscopy analysis, the cells processed as above were fixed using 2.5% glutaraldehyde, stained using Live/Dead staining and viewed under fluorescence microscope. For determining the viability count of adhered bacteria on the cell line, 0.1% of TritonX 100 was added to

lyse the cells in the microtitre plate. Hundred microliter of suspensions of untreated infected cells and different concentrations of bacteriocin treated infected cells were serially diluted with TSB medium, poured on agar plates and incubated for 24 h. After incubation, number of colonies was counted to compare with untreated samples.

Effect of Bacteriocins on *C. elegans*

Effect of bacteriocins was observed on *C. elegans* by using triplicate batches of 10 *C. elegans* for each bacteriocin concentration in a 24-well plate. Wells with S-medium containing bacteriocins but without worms and wells containing worms without bacteriocins were taken as positive and negative control, respectively (Bakkiyaraj and Pandian 2010). Synchronized L4 stage *C. elegans* were added to the 24-well plate and inoculated with bacteriocins in S-medium with *E. coli* OP50. *C. elegans* were treated with bacteriocins at 0.5XMIC, MIC and 2XMIC concentrations and incubated at 20 °C. Subsequently, the worms were scored for live or dead at 24 h and 48 h. A worm was regarded as dead when straight rigid structures appear and no body movement was observed. *C. elegans* from each bacteriocin treated and untreated wells was taken, washed thrice with M9 buffer, treated with 1% sodium azide and visualized under phase contrast microscopy (IX71 OLYMPUS, Singapore) for morphological changes in *C. elegans*.

Statistical Analysis

All experiments were performed independently in triplicates and repeated minimum three times. The individual means and mean ± standard error (SE) were calculated using Microsoft Excel 2010. The results were analysed using Student's *t* test and $p < 0.05$ value was considered as statistically significant.

Results

Purification and Characterization of Bacteriocins

Purified bacteriocin fractions BacF1 and BacF2 revealed total activity of 1280 and 640 AU/mL, respectively (Table S1) and showed single peaks at retention time 3.43 and 4.45 min, respectively in analytical RP-HPLC profile at $A_{280 nm}$ and purified bacteriocins BacF1 and BacF2 showed antibacterial activity against *S. aureus* MTCC 96 (Fig. 1c and d). BacF1 showed 16% bacteriocin recovery as compared to BacF2 with only 8% of bacteriocin yield.

Tris-tricine SDS-PAGE showed bands of purified bacteriocins obtained from semi-preparative RP-HPLC (Fig. 1). Dense separate gel bands were visible in Fig. 1a and b and

compared with the molecular weight marker. Figure 1a demonstrated clear band above molecular marker 3.5 kDa, whereas Fig. 1b showed a dense band between 1.06 and 3.5 kDa molecular weight. The molecular weight of bacteriocins, BacF1 and BacF2 were reported as 4.039 kDa and 1.6 kDa by Q-TOF ESI mass spectrometry in our previous study (Ray Mohapatra and Jeevaratnam 2019).

Antibacterial Activity

Antibacterial efficacy of purified bacteriocins BacF1 and BacF2 were determined against Gram-positive and Gram-negative pathogens, which were found to be potent against *Listeria monocytogenes*, *S. aureus*, *E. coli*, *Aeromonas hydrophila* bacterial strains (Table 1). BacF1 showed comparatively lower potential against *P. aeruginosa* strains, whereas BacF2 were less effective against Gram-negative bacteria such as *P. aeruginosa*, *E. coli* and *K. pneumoniae* strains (Table 1) compared to Gram-positive pathogens.

Effect of Protease Enzyme on Purified Bacteriocin

The purified bacteriocins BacF1 and BacF2 showed complete loss of activity when treated with protease enzyme and zone of inhibition was not observed against *S. aureus* MTCC 96 after protease enzyme treatment (Fig. S1), confirming their proteinaceous nature. Also, bacteriocins without enzyme treatment were taken as control which retained their antibacterial efficacy.

Determination of Minimum Inhibitory Concentration

Bacteriocins BacF1 and BacF2 showed > 90% inhibition of *S. aureus* MTCC 96 at 62.5 μM and 125 μM, respectively (Fig. 2). Nisin also showed 90–95% inhibition at 62.5 μM and 125 μM compared to BacF1 activity. The results suggested bacteriocins to be effective against *S. aureus* MTCC 96 and no significant difference between inhibitory concentration of bacteriocins, specifically BacF1 and Nisin was observed (Fig. 2). However, BacF2 showed higher inhibitory concentration against *S. aureus* MTCC 96 compared to BacF1.

Time-Kill Assay

Bactericidal activity of BacF1 and BacF2 was studied by killing assay on exponential growth of *S. aureus* MTCC 96 after treatment with bacteriocins and compared with untreated bacteria. Both BacF1 and BacF2 treated culture showed significant reduction in growth of the pathogen after 6 h of the treatment and gradually decreased till 12 h as compared to the control. Cell viability of treated samples
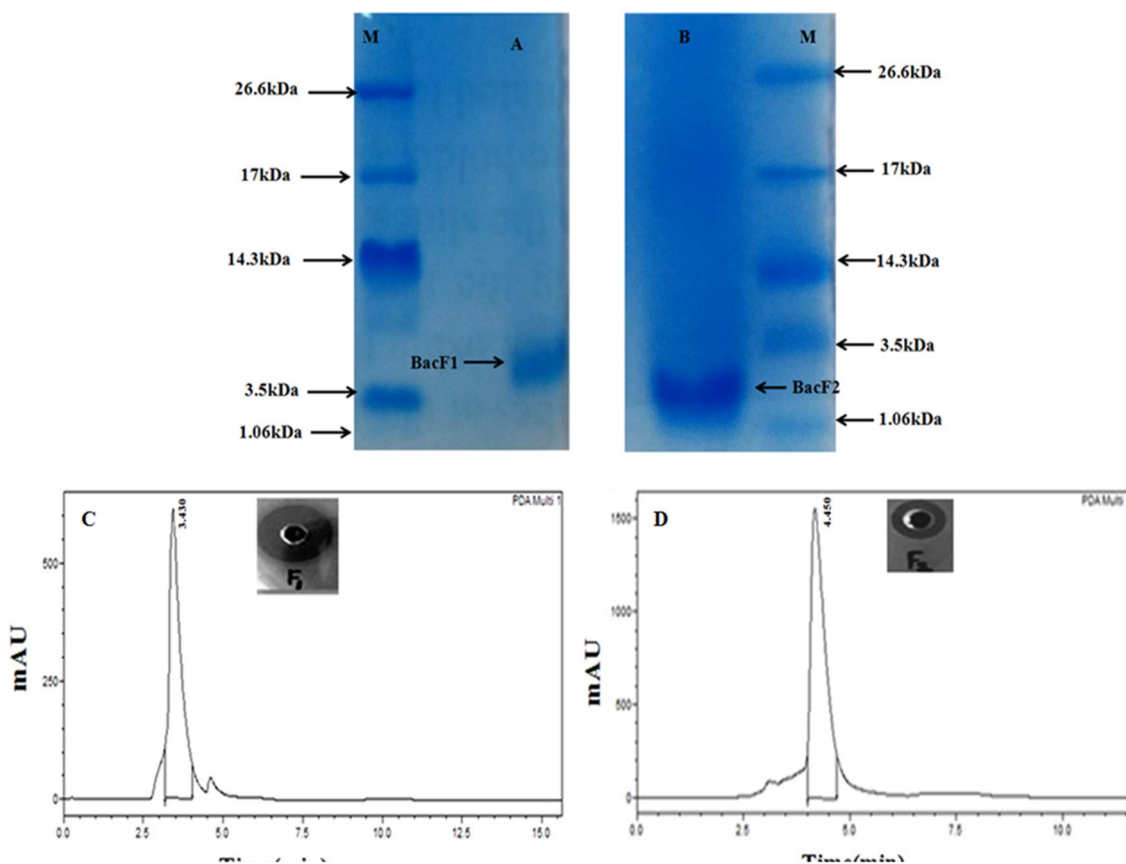
Fig. 1 Tricine SDS-PAGE of bacteriocins. **M** is the ultra-low range molecular weight protein marker. Gel bands shown by purified bacteriocins **a** BacF1 and **b** BacF2 Chromatographic analytical RP HPLC profile of purified bacteriocins monitored at 280 nm, **c** BacF1 and **d** BacF2 and their antibacterial activity against *S. aureus* MTCC 96
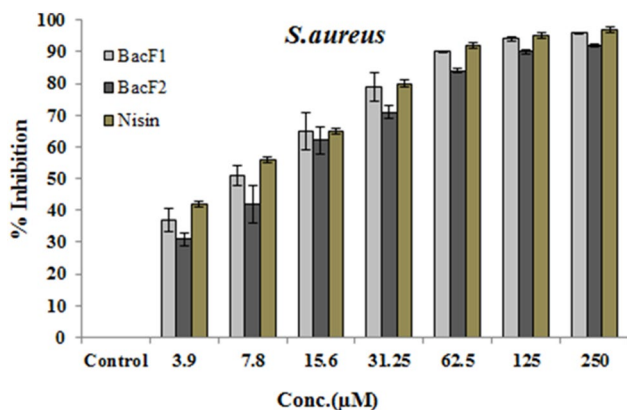

Fig. 2 Determination of minimum inhibitory concentration of purified bacteriocins BacF1 and BacF2 on *S. aureus*. Nisin was used as control. Results of three experiments performed independently in triplicates presented as mean ± standard error
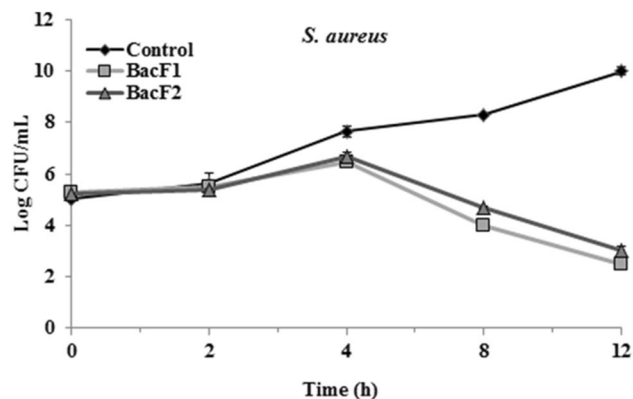

Fig. 3 Effect of purified bacteriocins BacF1 and BacF2 on growth and survival of *S. aureus*. BacF1 and BacF2 showed effect on *S. aureus* and calculated as colony forming units (CFU) after equal time intervals of incubation. Results expressed as mean ± standard error calculated from three independent experiments repeated in triplicates

was determined after 2 h of incubation but not much difference in viability was observed in comparison to the control sample (5 Log CFU/mL). Considerable reduction in CFU count of *S. aureus* MTCC 96 was found after 6 h and 8 h of treatment, nearly 50% of reduction in the cell viability was observed by BacF1 and BacF2 (Fig. 3). After 12 h of BacF1 and BacF2 treatment, the viability count was decreased to

2.5 and 3 Log CFU/mL, respectively from 10 Log CFU/mL of *S. aureus* MTCC 96 untreated culture (Fig. 3). However, no colonies survived the bacteriocin treatment after 12 h.

Scanning Electron Microscopy

The SEM results clearly displayed disrupted cell morphology in bacteriocin treated *S. aureus* MTCC 96 cells compared to the untreated ones. Untreated cells were intact, turgid and separated from one another as viewed under SEM (Fig. 4a), whereas cells treated with purified bacteriocins BacF1 and BacF2 at MIC (62.5 μM and 125 μM) were deformed and had prominent pores on the cell membrane (Fig. 4b and c). Scanning electron microscopic images clearly showed pore formations and leakage of cellular contents which confirmed the bactericidal nature of the bacteriocins.

Biofilm Assays

Congo Red Assay

Congo red agar (CRA) method showed *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 3541 strains that displayed black colonies on Congo red medium as distinctive biofilm producing strains but no dry crystalline morphology was observed (Fig. S2). However, *S. aureus* MTCC 737 and *P. aeruginosa* MTCC 2295 isolates showed red or pink colonies (Fig. S2). Only a few bacterial strains screened by CRA assay displayed red (pink) colonies at 37 °C after 24 to 48 h of incubation.

Antibiofilm Activity of Bacteriocins

S. aureus MTCC 96 and *P. aeruginosa* MTCC 3541 showed strong biofilm forming ability as analyzed by crystal violet staining method. Purified bacteriocins BacF1 and BacF2 significantly reduced biofilm formation by pathogens shown by qualitative (Fig. 5a and b) as well as quantitative crystal violet assay (Fig. 5c and d). BacF1 and BacF2 treated at

sub-MIC (31.25 μM) inhibited 70% and 65% of biofilms formed by *S. aureus* MTCC 96, respectively while 55% (Fig. 5c) and 50% of biofilm disruption was found in *P. aeruginosa* MTCC 3541 (Fig. 5d). Bacteriocins were more effective against *S. aureus* biofilms than on biofilms formed by *P. aeruginosa*. BacF1 was found to be more potent antibiofilm agent as compared to BacF2.

Bacteriocins significantly inhibited biofilm formation which was also evidently observed under fluorescence microscope and confirmed by SEM images (Figs. 6 and 7). These results were consistent with our findings of crystal violet assay.

Quantitative Real-Time PCR

Biofilm forming genes present in *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 3541 were suppressed by bacteriocins at sub-MIC (31.25 μM). The genes analyzed in this study demonstrated reduced expression in the treated groups as compared with the untreated conditions. The intercellular adhesion genes are virulence factors accountable for formation of biofilm in *S. aureus*. Similarly, *pelA*, *psl, rhlA* genes were found to induce polysaccharide and rhamnolipid production in *P. aeruginosa* shown in Fig. 8c and d. The genes *icaC*, *icaD* and *pelA*, *psl*, *rhlA* showed down-regulation of expression compared to untreated control upon treatment with bacteriocins. BacF1 was found to be more effective in suppressing the expression of *icaC* and *icaD* gene as compared to BacF2 after bacteriocin treatment of *S. aureus* biofilms (Fig. 8a and b). Relative quantitative expression of *pelA* gene was found to be more reduced than gene expression of *psl* and *rhlA*. *psl* and *rhlA* genes, which showed a suppressed expression but comparatively less than *pelA* gene. These results revealed that BacF1 and BacF2 treated conditions were considerably effective in down-regulating the expression of biofilm forming genes in *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 3541, also confirmed the antibiofilm activity of bacteriocins.

Fig. 4 Scanning electron micrograph images of *S. aureus* cells treated with bacteriocins. Purified bacteriocins activity on *S. aureus* cells by membrane pore formation (indicated by arrows) **b** BacF1 treated and **c** BacF2 treated cells as compared to **a** untreated *S. aureus* cells
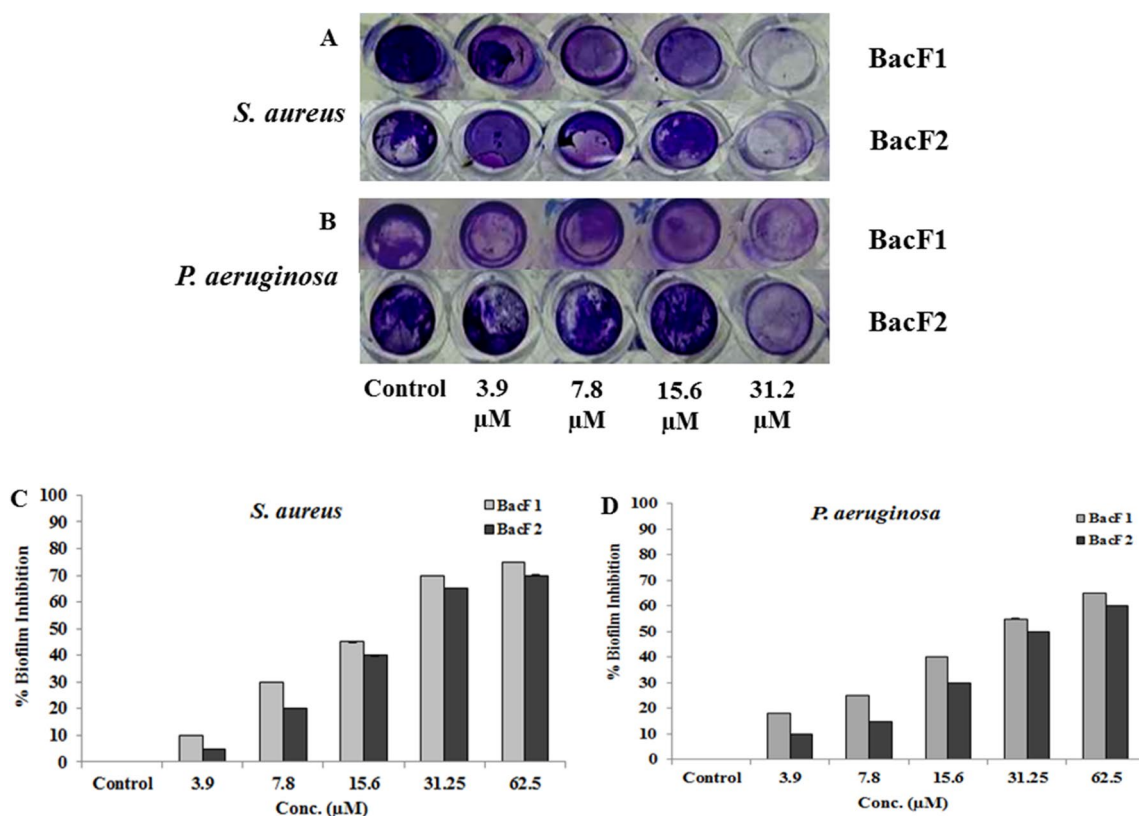
Untreated | Treated

BacF1 | BacF2
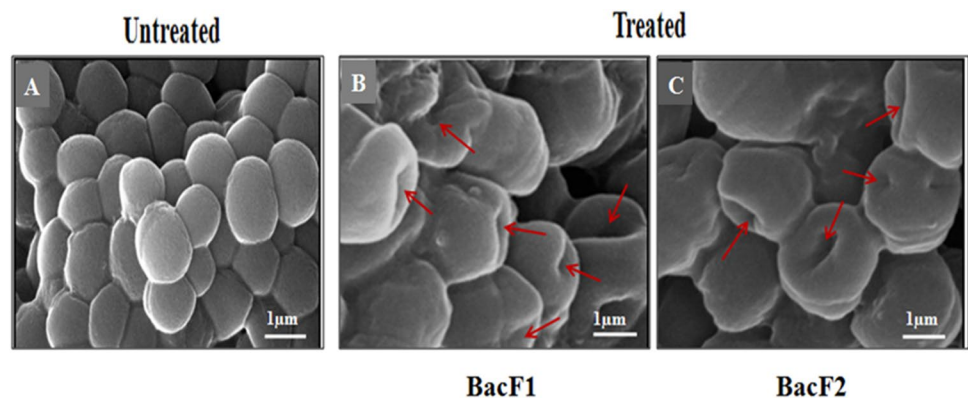
Fig. 5 Antibiofilm activity of purified bacteriocins BacF1 and BacF2 on biofilm formed by *S. aureus* and *P. aeruginosa*. Qualitative analysis of biofilm formation and inhibition by crystal violet stain against **a** *S. aureus* and **b** *P. aeruginosa*. Quantitative results of biofilm inhibition of **c** *S. aureus* and **d** *P. aeruginosa* biofilms using crystal violet assay. Results of experiments performed thrice in triplicates expressed as mean ± standard error
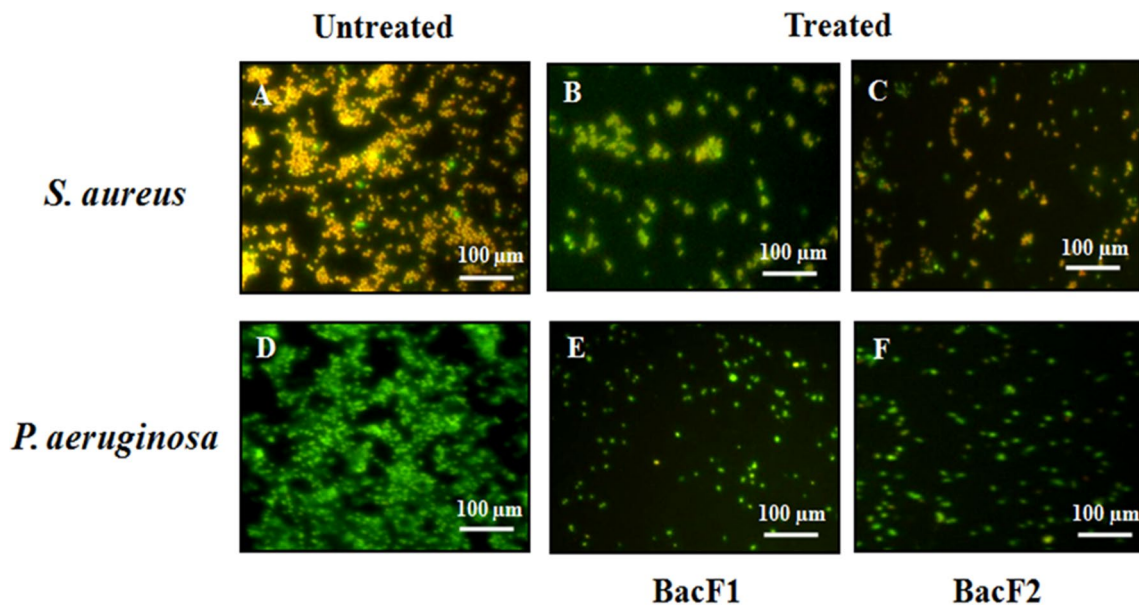

Fig. 6 Biofilms formed by *S. aureus* and *P. aeruginosa* and disruption of biofilms after treatment with purified bacteriocins as viewed under fluorescence microscope. **a** and **d** Untreated *S. aureus* and *P. aerugi-nosa* biofilms. **b** and **c** Bacteriocins treated biofilms of *S. aureus* and **e** and **f** *P. aeruginosa* biofilms treated with bacteriocins (31.25 μM)
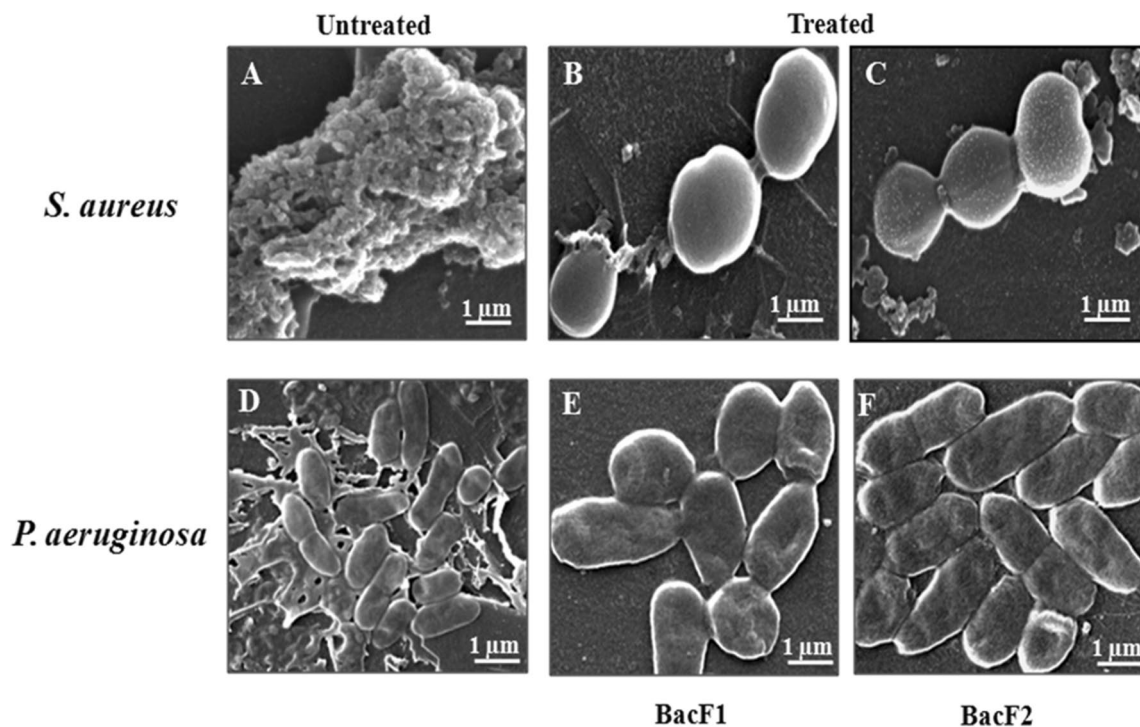
Fig. 7 Biofilms formation by *P. aeruginosa* and *S. aureus* and inhibition by purified bacteriocins viewed under scanning electron microscope. **a** and **d** Untreated biofilms formed by *S. aureus* and *P. aerugi-* *nosa*. Bacteriocins, **b** and **e** BacF1 and **c** and **f** BacF2 treated biofilms formed by *S. aureus* and *P. aeruginosa*

Fig. 8 Effect of BacF1 and BacF2 on the expression of biofilm forming genes in *S. aureus* and *P. aeruginosa* using real time PCR. Quantitative relative gene expression of biofilm adhesion in *S. aureus* and *P. aeruginosa* when supplemented with BacF1and BacF2 at sub-MIC (31.25 μM) compared with untreated control showed significant down-regulation of gene expression by both bacteriocins **a** and **b** *S. aureus* and **c** and **d** *P. aeruginosa*. The values expressed as mean ± standard error were calculated from experiments performed three times independently in triplicates
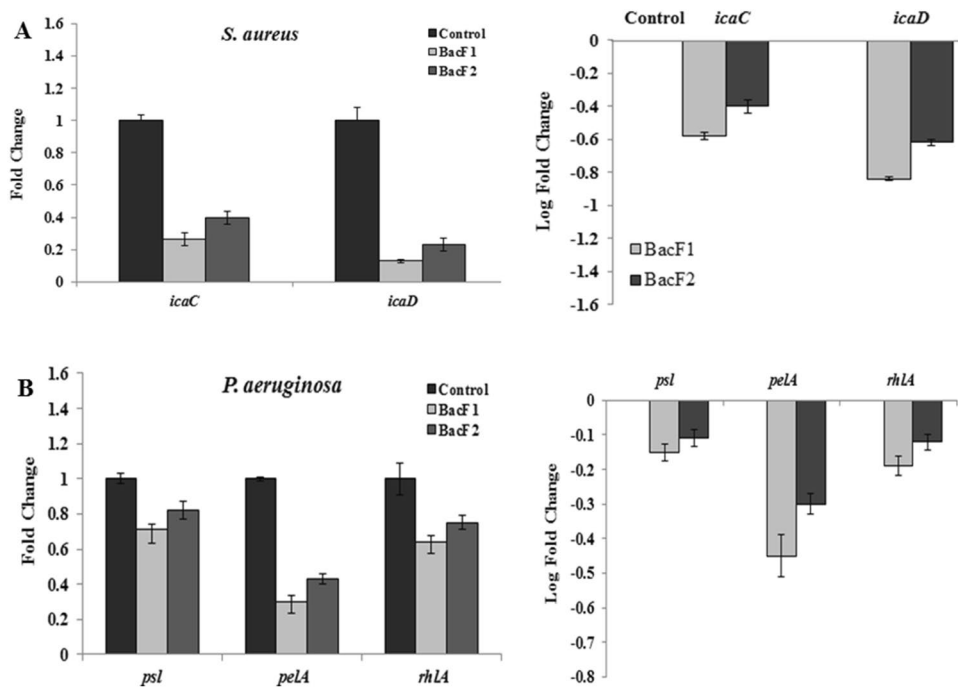
Cytotoxicity Assays

Effect of Bacteriocins on Cell Viability

Bacteriocins BacF1 and BacF2 were analyzed for cytotoxicity on HEK-293 cells by MTT assay. The viability increases as concentration decreases and at lower concentration i.e. sub-MIC (31.25 µM) both BacF1 and BacF2 showed > 90% of cell viability. Bacteriocins at 62.5 µM showed more than 80% of viability and very less cytotoxicity was observed. Only at higher concentration (500 µM) of BacF1 and BacF2, nearly 60% of cell viability was found as compared to untreated cells (Fig. S3). No significant cytotoxic effect was found on HEK-293 cells by bacteriocins BacF1 and BacF2.

Cell Adherence Assay

Cytotoxicity assay of bacteriocins on HEK-293 cell line by MTT assay showed very minimal cytotoxicity but only at higher concentrations. The effect of the bacteriocins on cytotoxicity induced by *S. aureus* MTCC 96 adhered to HEK-293 cells was analyzed by viable plate count and microscopic observation by Live/Dead staining under fluorescence microscopy. *S. aureus* MTCC 96 infected cells treated with BacF1 and BacF2 revealed improved survival rate and cell viability as compared to untreated infected control (Fig. 9a and b). Cells treated with BacF1 and BacF2 at MIC (62.5 µM) showed better survival and lower bacterial adherence than untreated infected control (Fig. 9b). However, BacF1 was found to be more effective even at lower concentrations in prevention of *S. aureus* MTCC 96 adherence to HEK-293 cells. The morphological changes in *S. aureus* MTCC 96 adhered and unadhered HEK-293 cells as well as bacteriocins treated bacteria adhered cells can be clearly seen by microscopic images (Fig. 9c). The difference in cell morphology of treated cells was also clearly visible compared to the adhered cells without bacteriocins treatment (Fig. 9c).
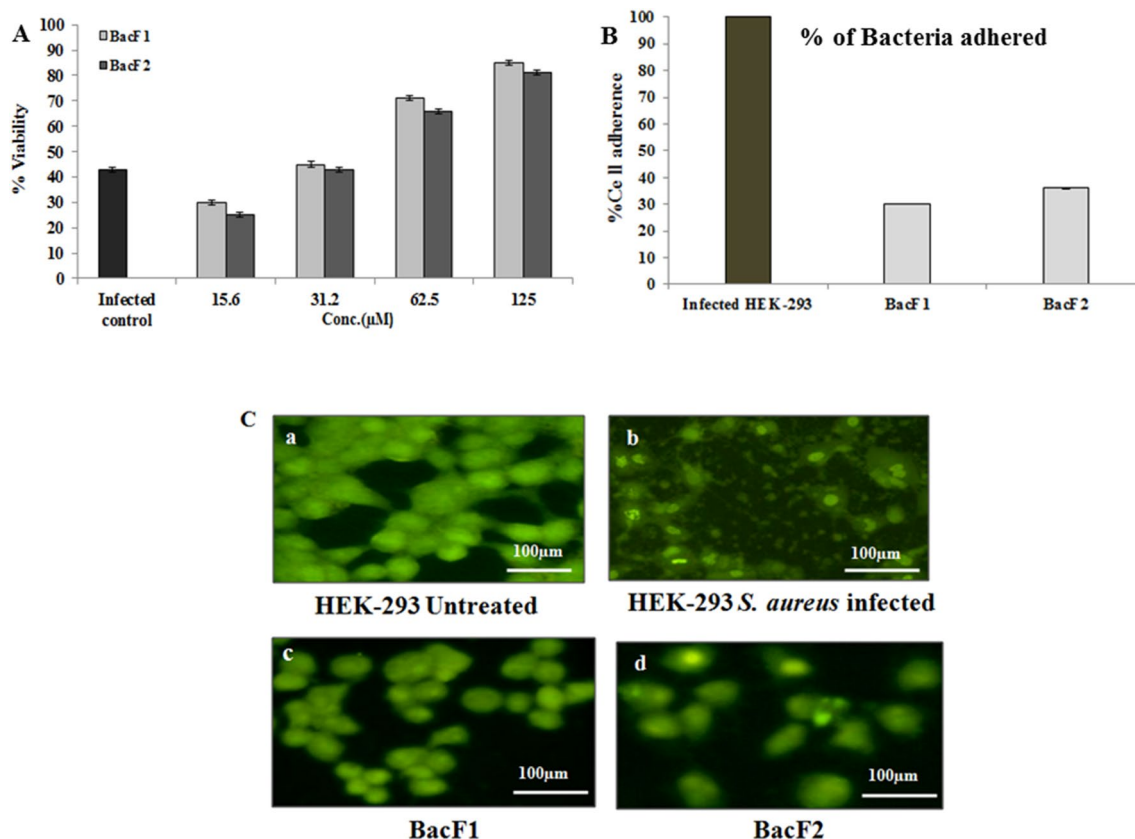

Fig. 9 Effect of purified bacteriocins BacF1 and BacF2 on *S. aureus* adhered mammalian cell line HEK-293. **a** Survival percentage of *S. aureus* infected HEK-293 cells treated with BacF1 and BacF2 by MTT assay taking infected control. **b** Viability assay by CFU count shows percentage of bacterial adherence to HEK-293 treated with BacF1 and BacF2 compared with infected control. **c** Fluorescence microscopic images showing morphological changes of (a) uninfected HEK-293 and (b) *S. aureus* infected cells without treatment (c) *S. aureus* infected cells with BacF1 and (d) BacF2 treatment at 62.5 µM

Effect of Bacteriocins on *C. elegans*

Effect of purified bacteriocins on wild type *C. elegans* was investigated until all the worms were found dead. Our results showed that *C. elegans* treated with bacteriocins BacF1 and BacF2 were alive with longer survival time. The worms were viable for more than 48 h after the bacteriocins treatment. After incubation, no significant cytotoxicity was found on *C. elegans* and also showed undamaged morphology at different time intervals (Fig. S4). The microscopic images clearly demonstrated live worms which maintained sinusoidal position and showed body movements after treatment with BacF1 and BacF2 at 24 h and 48 h as compared with untreated worms which were only provided with live *E. coli* OP50 bacteria without any bacteriocins (Fig. S4). Moreover, the treated *C. elegans* also showed survival in different concentrations of bacteriocins at specific time periods.

Discussion

This study mainly focuses on characterization and evaluation of two bacteriocins produced by *Lactobacillus plantarum* SJ33 strain. The purified bacteriocins BacF1 and BacF2 were recovered from semi-preparative RP-HPLC. Our result was also supported by previous reports, demonstrating bacteriocins from *L. plantarum*, *L. acidophilus* and *L. fermentum* (Saranya and Hemashenpagam 2013; Zhao et al. 2015). Tris-Tricine SDS-PAGE analysis showed the presence of two separate gel bands for bacteriocins BacF1 and BacF2 which were also demonstrated in our previous study (Ray Mohapatra and Jeevaratnam 2019). These results were in agreement with earlier studies on bacteriocins produced from LR/14 strain (Tiwari and Srivastava 2008) and bacteriocins produced by various LAB strains such as *Lactobacillus sakei*, *Leuconostoc mesenteroides*, *Carnobacterium piscicola* and *Enterococcus faecium* which reported production of more than one bacteriocin (Sawa et al. 2013). Here, BacF1 exhibited a broad range of antibacterial activity on Gram-positive and Gram-negative pathogens, *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa*, whereas BacF2 was more effective against Gram-positive pathogens as compared to Gram-negative bacteria. The partially purified bacteriocin preparation obtained previously also exhibited a wide array of antibacterial activity against various Gram-positive and Gram-negative bacteria. The bacteriocin preparation demonstrated combinatorial activity of BacF1 and BacF2 which were present in a ratio of 2:3 as revealed by RP-HPLC data in our earlier study (Ray Mohapatra and Jeevaratnam 2019). Few bacteriocins from *Lactobacillus* spp. were reported earlier showing better antibacterial activity on Gram-negative bacteria (Todorov and Dicks 2005),

whereas other bacteriocins could not inhibit Gram-negative bacteria (Jiang et al. 2012; Miao et al. 2014). In this study, no activity was observed after the treatment of protease enzyme (1 mg/mL) on purified bacteriocins and complete loss of antibacterial activity was observed that confirmed proteinaceous nature of bacteriocins. Previous study also suggested loss of activity by bacteriocin-like compound from *L. plantarum* TF711 strain with proteases (Hernández et al. 2005). Similar activity was also observed in bacteriocins produced by *Pediococcus acidilactici* and other bacteriocins from various *Lactobacilli* strains which were reported to produce proteinaceous antimicrobial compounds (Leroy and De Vuyst 2010; Pal et al. 2010; Vidhyasagar and Jeevaratnam 2013) and antimicrobial substances those remained unaffected by treatment with lipase and α-amylase (Todorov and Dicks 2009; Moh et al. 2015). Whereas, some bacteriocins such as BAC-IB17 were reported as protease resistant in nature and showed stability against various proteolytic enzymes (Ansari et al. 2018).

Purified bacteriocin, BacF1 inhibited the growth of *S. aureus* MTCC 96 at 62.5 μM, whereas BacF2 showed inhibition at 125 μM, proving BacF1 to be more effective than BacF2. An earlier study reported Plantaricin K25 from *L. plantarum* showing MIC of 125 and 250 μg/mL against different pathogenic strains (Wen et al. 2016) and other study on bacteriocin, caseicin from *L. casei* showed higher MIC of 5 mg/mL and 2.5 mg/mL on *E. coli* and *S. aureus* pathogens (Lü et al. 2014). According to these previous reports bacteriocins of our study were found to be more potent against various pathogens than the previously characterized and identified bacteriocins. Most of the bacteriocins such as Plantaricin 423 and Amylovorin L471 showed bactericidal effect, whereas other bacteriocins such as plantaricins and bacteriocin-like substances were found to be bacteriostatic in nature (Reenen et al. 1998; Callewaert et al. 1999). Bacteriocins with bactericidal activity showed rapid effect on pathogens after treatment. However, time-dependent assay demonstrated effect of bacteriocins within 6 h of treatment and also showed decrease in viable CFU count in *S. aureus* after 4–6 h of bacteriocins treatment. Bacteriocin KBC11 produced by *Lactobacillus plantarum* strain from a previous study also showed reduction of pathogen growth after 6 h of the treatment, whereas no colonies were found after 12 h of incubation (Sadishkumar and Jeevaratnam 2018) which supported our results.

BacF1 and BacF2 showed membrane disruption and pore formation on *S. aureus* cells indicating leakage of cellular contents. Scanning electron micrographs clearly showed deformation and damage of cell morphology, thus confirming the bactericidal nature of purified bacteriocins. Previous studies on bacteriocins, such as BAC-IB17 and Bac-IB45 demonstrated bactericidal action on the indicator strains that may be due to increase in membrane permeability that

leads to pore formation and subsequently causes cell death (Ansari et al. 2018; Ibrahim et al. 2020). The above data supported the results of our study. The bactericidal effect of each bacteriocin from various LAB strains may vary against different pathogenic strains i.e., bacteriocins can be bacteriostatic or bactericidal in nature (Dimitrov et al. 2010). Few bacteriocins were reported to be bacteriostatic against specific pathogenic strain while other bacteriocins showed bactericidal activity against other pathogens (Tiwari and Srivastava 2008). The most common mechanism of action of bacteriocin was recognized as pore formation leading to leakage of cellular materials from target cells (Héchard and Sahl 2002; Hammami et al. 2011).

Biofilms have become a major cause of health related problems, thus new antibiotics are urgently required to reduce the growing hazard from biofilm forming pathogens that are resistant to commercial antibiotics (Roe et al. 2018). This study evaluated the biofilm formation in pathogens by Congo red assay which screened the bacterial strains and was simple to perform. The CRA analysis was commonly based on color of bacterial colonies formed on the agar plates, ranging from pink or red for non-biofilm forming bacteria to black colonies for biofilm formers (Kaiser et al. 2013). The study revealed potential antibiofilm activity of purified bacteriocins against pathogens, *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 3541 which were classified as strong biofilm formers based on crystal violet assay. BacF1 and BacF2 showed potential antibiofilm activity against *S. aureus* than *P. aeruginosa* strain at sub-MIC (31.25 μM). A novel bacteriocin, Sonorensin was reported earlier that showed inhibition of biofilms formed by *S. aureus* strain (Chopra et al. 2015). As per previous studies bacteriocins such as lacticin Q and nisin A also exhibited antibiofilm potential against methicillin-resistant *Staphylococcus aureus* (Okuda et al. 2013).

The quantitative expression studies demonstrated suppression of biofilm adhesion genes *icaC* and *icaD* in *S. aureus* by purified bacteriocins whereas only *pelA* gene expression was down-regulated considerably than *psl* and *rhlA* genes when compared to control in case of *P. aeruginosa*. Although previous studies have supported the presence of intercellular adhesion genes and *icaADBC* dependent pathway in *S. aureus* (Rachid et al. 2000; Ong et al. 2019) but *ica*-operon independent pathways were also found in some of the *Staphylococcus* species. Moreover, few studies on polyphenols were reported to have inhibitory potential to disrupt biofilms formed by *S. aureus* isolates at sub-minimal concentration (Blanco et al. 2005). A previous study also reported the effect on extracellular polysaccharide production in *P. aeruginosa* by using plant extracts and antibiotics (Lakshmanan et al. 2019). Additionally, polyphenols were also known as anti-virulence compounds that attenuated and down-regulated the production of EPS at sub-MIC, thus

possessing the ability to control and modulate the quorum-sensing pathway in *P. aeruginosa* (Yin et al. 2015).

The cytotoxic study of purified bacteriocins showed no adverse effect on HEK-293 cell line even at higher concentration (4XMIC) and only showed mild cytotoxicity at higher concentration (500 μM). The study showed no significant cytotoxicity on HEK-293 cells by BacF1 and BacF2. Previous study on bacteriocins from *Bacillus* spp. also showed 91% cell viability on HEK-293 cells and Plantaricin A from *L. plantarum* showed toxicity at 10–100 μM concentration range where no toxic effect was seen on normal as well as cancer cells below 10 μM concentration (Sand et al. 2010). Another recent report on bacteriocin BAC-IB17 showed anticancer activity on HeLa cells but no cytotoxicity was found against normal cells and showed 90% of cell viability (Ansari et al. 2020). Similarly, BacIB45 produced by *Lactobacillus plantarum* revealed 85% cell viability at inhibitory concentration after bacteriocin treatment (Ibrahim et al. 2020), which supported this study. Few earlier studies also reported further characterized bacteriocins with less potential activity than bacteriocins of our present interest. Nisin and pediocin demonstrated lower cytotoxic effect on normal Vero cell lines (Vaucher et al. 2010). Additionally, Nisin and other lantibiotics showed mild cytotoxic effect on various eukaryotic cell lines at higher than lethal concentration (Griffiths et al. 2011). Bioactive metabolites produced by *L. plantarum* showed low cytotoxicity on normal mammalian cells (Chuah et al. 2019). Bacteriocins have been reported for their variable effect on mammalian cell lines due to different unknown factors and the actual mechanism involved in cytotoxicity is yet to be fully explained. Purified bacteriocins reported in this study also improved the survival of *S. aureus* adhered HEK-293 cells. The adhered cells treated with BacF1 and BacF2 showed better survival at inhibitory concentration that killed the bacteria. Morphological changes of bacteria adhered cells treated with bacteriocins were clearly visible under fluorescence microscope using Live/Dead staining. Previous reports suggested *Lactobacillus* strains to reduce cytotoxicity as well as attachment of bacteria to the normal epithelial cells and human intestinal epithelial cell line was treated with Plantaricin P1053 which was found to induce cell viability of CCD841, a normal cell line (Giani et al. 2019) which supported our results. Very few studies were found to be reported on human embryonic cells and the effect of bacteriocins on pathogen interaction and bacterial adherence to the normal mammalian cells.

Further, our findings have clearly displayed that *C. elegans* treated with bacteriocins exhibited better survival with intact morphology and no significant cytotoxicity. Results of a previous study showed that *C. elegans* infection liquid assay assists in better identification of potential bacteriocins

than in vitro diffusion assays (Son et al. 2016). In earlier studies, *C. elegans* was only used as a toxicity indicator for screening heavy metals, organic solvents, environmental pollutants and toxins (Moy et al. 2006). Though *C. elegans* is one of the common model used for biological experiments but only few studies have focused on the effect of antimicrobial substances on survival and growth of the nematode (Niu et al. 2016).

The above study characterized and analyzed two bacteriocins which showed significant antibiofilm potential and broad spectrum activity. The bacteriocins effectively inhibited bacterial adherence on normal mammalian cell line and enhanced the cell viability without adversely affecting the mammalian cells.

This work reported purification of two bacteriocins from *Lactobacillus plantarum* SJ33, which were bactericidal, antibiofilm and non-cytotoxic. The yields of bacteriocins were low and were sensitive to protease. The combination therapies of bacteriocins with traditional antibiotics can be used as a better antimicrobial and antibiofilm agent with potential activity even at lower doses. The complete characterization of bacteriocins, their mechanism of action and evaluation of the mechanism of action associated with survival of *C. elegans* upon treatment of antimicrobial agents are the limitations of this study. The increased knowledge in these areas can improve the effectiveness of bacteriocins in pharmaceutical applications.

Conclusions

This study characterized two purified low molecular weight bacteriocins produced by *Lactobacillus plantarum* SJ33 that possess significant properties such as broad spectrum antibacterial and antibiofilm activity against biofilm forming pathogens. Purified bacteriocins showed antibacterial activity against various pathogens and bactericidal effect on *S. aureus* cells was confirmed by SEM analysis that revealed membrane pore formation. Antibiofilm potential of purified bacteriocins on *S. aureus* and *P. aeruginosa* was confirmed through fluorescence and scanning electron microscopic images. Bacteriocins remarkably down-regulated the expression of *ica* genes in *S. aureus* and *pelA*, *psl*, *rhlA* genes in *P. aeruginosa*, respectively. Bacteriocins improved viability of *S. aureus* adhered normal mammalian cells with no considerable cytotoxicity at inhibitory concentration. *C. elegans* was used as a direct tool for assessing the potential of bacteriocins and can also facilitate the analysis of more prospective bacteriocins in future. The study highlights the possible medical applications of bacteriocins that opened new avenues in healthcare industry.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval This manuscript does not contain any research involving any human and/or animal participants.

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