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Antibacterial and Synergistic Activity Against β-Lactamase-Producing Nosocomial Bacteria by Bacteriocin of LAB Isolated From Lesser Known Traditionally Fermented Products of India

Koel Biswas, Supriya Upadhayay, George Freddyleen Rapsang, Santa Ram Joshi*

Microbiology Laboratory, Department of Biotechnology & Bioinformatics, North-Eastern Hill University, Shillong, Meghalaya, India.

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ABSTRACT

There is an ever-growing need to control antibiotic-resistance owing to alarming resistance to commonly available antimicrobial agents for which contemporary and alternative approaches are being explored. The present study assessed the antibacterial activity of bacteriocins from lactic acid bacteria (LAB) from lesser known traditionally fermented products of India for their synergistic potential with common antibiotics against clinical β-lactamases producing pathogens. A total of 84 isolates of LAB were screened for their antibacterial efficacy against *Streptococcus pyogenes, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae* and *Bacillus cereus* as well as against clinical pathogens harbouring β-lactamase genes such as bla_{CTX-M}, bla_{VIM}, bla_{IMP}, bla_{SHV} and bla_{NDM}. Synergistic activity of bacteriocins were determined in combination with antibiotics namely, cefotaxime, polymyxin B, imipenem and tigecycline. Purified bacteriocins from *Lactobacillus*, *Pediococcus* and *Enterococcus* inhibited the growth of β-lactamase harbouring clinical pathogens which significantly higher inhibitions when compared with antibiotics alone. Minimum inhibitory concentration of the extracts ranged from 6.66 to 26.66 mg/ml and 10 to 33.33 mg/ml for *Pediococcus pentosaceus* LU11 and *Lactobacillus plantarum* LS6. The bacteriocinogenic activity of LAB opens scope for bioprospection of antibacterial components in the current struggle against increasing pandrug resistance and slowing down the expansion of multi-drug resistance.

1. Introduction

Nosocomial infections have become a prime concern in the past few decades. A nosocomial can be defined as an infection acquired in hospital by a patient admitted for a reason other than that infection (Ducel *et al.* 2002) Microorganisms such as *Staphylococcus aureus*, coagulase-negative staphylococci, enterococci and those belonging to the Enterobacteriaceae family are found to be the source of such hospital acquired infections. The upsurge in bacterial resistance towards antibiotics and appearance of multidrug-resistant (MDR) microorganisms has been an important factor for increasing nosocomial infections. Pathogens responsible for such infections are found to be resistant to single or multiple antibiotics, which demands development of new antimicrobials. In the recent past, variety of compounds possessing antimicrobial property such as plant-derived compounds, bacteriophages and potential bactericidal compounds from bacteria have been investigated for their activity against such MDR pathogenic strains (Singh *et al.* 2010; Sulakvelidze *et al.* 2001). Antimicrobial peptides known as bacteriocins have attracted attention in this regard in the recent past (Rapsang *et al.* 2011).

Bacteriocins from lactic acid bacteria (LAB) are ribosomally synthesized antimicrobial peptides or proteins which possess inhibitory activity towards wide range of pathogens (De Vuyst & Leroy 2007). LAB are non-pathogenic probiotic microorganisms, present in normal flora which exert positive influence in host physiology and belong to the genus *Lactobacillus, Enterococcus, Pediococcus* and *Bifidobacterium* (Martha *et al.* 2001; Ouwehand *et al.* 2002). Studies have established the inhibitory potential of probiotics against pathogenic strains of *Salmonella*, *Escherichia*, *Staphylococcus, Bacillus, Klebsiella* and *Listeria* (Drago *et al.* 1997; Rapsang and Joshi 2012; Rapsang *et al.* 2013). These probiotics produce bacteriocins and bacteriocin-like molecules, which display a wide range of antimicrobial activity against Gram-positive and Gram-negative bacteria (De Vuyst & Leroy 2007).

* Corresponding author.
E-mail address: srjoshi2006@yahoo.co.in (S.R. Joshi).
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With the evolution of bacterial resistance to vast range of antibiotics in humans, such probiotic therapy appears to be futuristic alternative to prevent dissemination of MDR pathogens. The inherent properties such as low toxicity, potency, availability of broad and narrow spectrum peptides of bacteriocins imply that they are viable alternatives to antibiotics (Cotter *et al.* 2013). Studies on lantibiotics such as nisin, lacticin 3147 belonging to the class I bacteriocin, have shown prominent activity against *Streptococcus pneumoniae*, staphylococci including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (Piper *et al.* 2009). Bacteriocin produced by particular strain of *Enterococcus faecium* against MDR bacterial species have been reported (Ali *et al.* 2012). The effectiveness of individual bacteriocins from potential LAB strains could be further enhanced through combination with other antimicrobials and commercially available antibiotics. Studies have reported the synergistic activity of nisin with antibiotics polymyxin E and clarithromycin against *Pseudomonas aeruginosa* (Giacometti *et al.* 2000) and with ramoplanin and other non−β-lactam antibiotics against various strains of MRSA and vancomycin-resistant enterococci (Giacometti *et al.* 2000; Brumfitt *et al.* 2002).

Large variety of traditionally fermented products produced and consumed by tribals of Northeast India are rich sources of LAB (Thapa *et al.* 2004; Tamang *et al.* 2005; Jeyaram *et al.* 2010; Rapsang *et al.* 2011; Taheri *et al.* 2011) and can be good source of probiotics and bacteriocins. Bacteriocin like compounds from LAB associated with traditional fermented foods to control the propagation of clinical and food borne pathogens with probiotic therapy appears to be an innovative alternative to reduce dissemination of pathogens.

The present study was aimed to screen bacteriocin-producing LAB from two traditionally fermented products *Tungtap* (fish product) and *Tungrymbai* (soybean product) and determine their antibacterial activity alone and in combination with commercially available antimicrobial agents against MDR pathogens and clinical isolates.

2. Materials and Methods

2.1. Sample collection

Fifteen samples of *Tungtap*, a traditionally fermented *Puntius* species (Figure S1) and eight samples of *Tungrymbai*, a traditionally fermented soybean (Figure S2) were collected (during 2012−2015) from retail markets of Northeast India in sterile containers and were transported aseptically to the laboratory for analysis within 24 h.

2.2. Isolation of LAB

The samples were homogenized using mortar and pestle. Ten grammes of the homogenised sample was then blended in 90 mL of 0.85% (w/v NaCl) physiological saline. The total viable LAB counts in each sample was analysed by spread plating the serially diluted samples on de Man, Rogosa and Sharpe (MRS) agar (HiMedia, Mumbai, India). The inoculated plates were incubated at 37°C for 48−72 h for isolation of LAB (Rapsang & Joshi 2012).

2.3. Test organisms

Reference strains, *Streptococcus pyogenes* MTCC 1925, *Enterococcus faecalis* MTCC 2729, *Escherichia coli* MTCC 730, *Klebsiella pneumoniae* MTCC 109 and *Bacillus cereus* MTCC 430 used for the study were obtained from Institute of Microbial Technology (Microbial Type Culture Collection Centre, Chandigarh, India). Apart from the reference strains, clinical pathogenic isolates producing different class of β-lactamase enzymes (extended-spectrum β-lactamases, carbapenemases) were also tested. The clinical pathogens included *K. pneumoniae* (KP7, KP6) harbouring $bla_{\text{CTX-M}}$, bla_{NDM}, *Acinetobacter baumannii* (AB13775) harbouring bla_{NDM}, *P. aeruginosa* (PA) harbouring bla_{NDM}, bla_{VIM}, bla_{IMP} and other MDR strains of *E. coli* (EC, IB9, GN13, GN9) harbouring $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{NDM} (Kumarasamy *et al.* 2010; Upadhaya and Joshi 2015). The sensitivity patterns of these organisms against standard antibiotics were previously tested using CLSI guidelines 2013 (Wayne 2013).

2.4. Screening of LAB for antimicrobial activity

Agar spot assay test (Schillinger & Lücke 1989) was carried out in triplicate to screen the LAB isolates for their antimicrobial activity. Each of the LAB isolate was grown in MRS broth for 24 h at 37°C. The cultures were then adjusted 1.5×10^8 CFU/mL. Ten microlitres of the cell suspension from each isolate was spotted onto the surface of MRS agar plates and incubated at 37°C for 24 h. After the incubation period, the colonies were then overlaid with 10 mL of Brain Heart Infusion (HiMedia, Mumbai, India) soft agar containing 10^7 CFU/mL of indicator organisms (*S. pyogenes, E. faecalis, E. coli, K. pneumoniae* and *B. cereus*). The plates were incubated overnight at 37°C before recording the diameter of inhibition zones.

2.5. Bacteriocin production and activity

The bacterial colonies showing antibacterial activity were selected as potential bacteriocin producers. The colonies were subcultured in 50 mL MRS broth and incubated for 24 h at 37°C in a rotary shaker with agitation at 150 rpm. The cultures were centrifuged at 10,000 rpm for 30 min at 4°C. A portion of the cell-free supernatant was neutralized with 1M NaOH (pH 6.8 ± 0.2) to rule out possible inhibition effects due to organic acids and filtered sterilized through 0.22 μm membrane (Puradisc 25mm, India). To determine the activity of the supernatant, agar well-diffusion method was performed in triplicate (Singh *et al.* 2010). Müller-Hinton agar (HiMedia, Mumbai, India) plates were swabbed with bacterial cell suspension of the indicator strains and clinical pathogens adjusted to 1.5×10^8 CFU/mL. Five-millimetre diameter wells were cut in the agar plate using a sterile cork borer. Hundred microlitre aliquot of each supernatant was added to each well and the plates were incubated at 37°C for 24 h. After incubation, zones of inhibition around the wells were measured manually.

2.6. Physio-chemical characterization of inhibitory substances

2.6.1. Effect of enzymes

Aliquots of 200 μL of the cell-free supernatant were treated with proteolytic enzymes such as catalase (2.6 U/mg), pepsin (16 U/mg), proteinase K (3 U/mg), pronase E (22 U/mg) and trypsin (15 U/mg). The assays were performed at a final concentration of 1 mg/mL and at pH 6.5, except for pepsin (pH 3.0). Treated samples were held at 37°C for 6 h. After the incubation process, samples were boiled at 100°C for 5 min to stop the reaction. Untreated cell-free supernatant was used as control. The antimicrobial activity of the treated and untreated supernatant was determined by agar well-diffusion assay following the above-mentioned protocol using the selected indicator strains.

2.6.2. Effect of temperature and different pH

To establish the effect of temperature on antibacterial activity, aliquots of 200 μL of the cell-free supernatant were heated at 60°C, 80°C and 100°C for 60 min and at 120°C for 15 min. Aliquots of 200 μL of the cell-free supernatant were adjusted to different pH from 2 to 10 with HCl (5 M) and NaOH (5 M) and incubated for 4 h at 30°C. The residual activity was tested as described previously.

2.7. Preparation of LAB extracts

To prepare the extracts, the LAB cultures were grown at 37°C for 48 h. Cell-free supernatant obtained by centrifuging the culture

broth at 10,000 rpm for 30 min was concentrated in rotary evaporator for 3 h at 30°C. The weight of the concentrated sample was determined and re-suspended in 0.05 M potassium phosphate buffer (pH 7) to make desired concentration and stored at −20°C until further use.

2.8. Evaluation of synergistic effect

The efficacy of LAB bacteriocin extract alone and in combination with commercially available antibiotics polymyxin B, tigecycline, imipenem and cefotaxime (HiMedia, Mumbai, India) against the MDR clinical pathogens was determined by using disc diffusion method in triplicate. To study the combined effect of bacteriocin extract and antibiotics, 20 μL (2 mg) of the bacteriocin extract was added to the standard antibiotic disc which was placed on Müller-Hinton agar plates inoculated with the test organisms. For positive control, standard antibiotic discs were used. The plates were incubated overnight at 37°C and zones of inhibition were recorded. The increase in fold area was assessed by calculating the mean surface area of the zone of inhibition obtained by the antibiotic alone and in combination with the bacteriocin (Birla *et al.* 2009). The fold area increase for the antibiotics was calculated by the formula $(b^2 - a^2)/a^2$, where a and b are the zone of inhibition for antibiotic alone and antibiotic in combination with bacteriocin, respectively.

2.9. Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of the LAB extracts was performed in triplicate using broth microdilution method (Nath & Joshi 2014) in 96 multiwell microtiter plates in triplicates. 0.5 McFarland standard suspension of the indicator bacteria and the clinical isolates were inoculated in Müller-Hinton broth medium. Fifty microlitres of the inoculum was added to appropriate wells containing 50 μL of bacteriocin extract of different LAB in different concentrations. The final concentrations of the extracts used to evaluate the antibacterial activity were in the range of 3.33−66.66 mg/mL. The 96-well plates were then sealed with parafilm and incubated in aseptic condition at 37°C for 18 h. One hour before the incubation period, 50 μL of 0.5% solution of 2,3,5-triphenyl tetrazolium chloride (Sigma, India) was added to the wells and the plates were incubated for another hour in dark conditions. The colourless tetrazolium salt was reduced to a red colour compound by biologically active organisms. The inhibition of growth can be detected when the solution in the well remains clear after the incubation with triphenyl tetrazolium chloride. The lowest concentration of each extract showing no visible growth was recorded as the MIC.

2.10. Identification of bacteriocin-producing LAB

The bacterial isolates were characterized based on colony morphology, Gram staining reaction and biochemical tests like catalase, oxidase activity, indole production, MR-VP, nitrate reduction, urease activity, citrate utilization and gelatin hydrolysis. Whole genomic DNA was extracted using HiPurA Bacterial and Yeast Genomic DNA Isolation Kit (HiMedia, Mumbai, India). The polymerase chain reaction was carried out using universal primer sets 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACTT-3′) (Thomas 2004) obtained from IDT, India, and amplified products were purified using QIAquick Gel Extraction Spin Kit (QIAGEN, Germany). The purified 16S rRNA gene products were sequenced using BigDye Terminator reaction method using Genetic Analyzer ABI 3130XL (Applied Biosystems, USA). BLAST programme (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed for the analysis of 16S rRNA gene sequence to determine the sequence homology against the

Table 1. Antagonistic activity of the LAB isolates against indicator bacteria and clinical pathogens

Reference strain	Isolated lactic acid bacteria										
	LS9	LS5	GS4	KB10	KTP1	KTP 5	KTP6	LS3	LS8	LU11	LS6
Streptococcus pyogenes MTCC 1925	10.6 ± 0.334	15.3 ± 0.334	17.6 ± 0.334	13.3 ± 0.667	11 ± 0.0	12.3 ± 0.667	11.6 ± 0.334	11 ± 0.0	10.6 ± 0.334	16 ± 0.0	–
Enterococcus faecalis MTCC 2729	10 ± 0.0	26.3 ± 0.334	25.3 ± 0.334	19 ± 0.0	15 ± 0.0	15.3 ± 0.334	17.6 ± 0.334	12.3 ± 0.667	14 ± 0.0	21 ± 0.0	–
Escherichia coli MTCC 730	11.3 ± 0.334	11.6 ± 0.334	18.3 ± 0.334	19 ± 0.334	12 ± 0.0	13 ± 0.0	14.3 ± 0.334	16.3 ± 0.334	10.6 ± 0.334	23 ± 0.0	11.6 ± 0.334
Klebsiella pneumoniae MTCC 109	10 ± 0.334	16 ± 0.0	19 ± 0.334	17 ± 0.0	10 ± 0.578	15.3 ± 0.334	15.3 ± 0.334	12.6 ± 0.334	11.3 ± 0.334	15 ± 0.0	10 ± 0.0
Bacillus cereus MTCC 430	12 ± 0.0	15 ± 0.0	13.3 ± 0.667	18 ± 0.0	14 ± 0.0	14 ± 0.0	11.3 ± 0.334	14 ± 0.0	–	16 ± 0.0	12.6 ± 0.334
Clinical pathogens											
E. coli GN17	–	18 ± 0.0	15.3 ± 0.334	10.3 ± 0.334	10 ± 0.578	10.3 ± 0.334	–	17 ± 0.0	–	17.6 ± 0.334	11.3 ± 0.334
K. pneumoniae KP7	–	16 ± 0.0	12.6 ± 0.334	–	–	–	–	17 ± 0.0	–	21 ± 0.0	18 ± 0.0
E. coli GN4	–	10.6 ± 0.334	10.6 ± 0.334	–	10.3 ± 0.334	12 ± 0.0	11.6 ± 0.334	11.3 ± 0.334	11.3 ± 0.334	10.6 ± 0.667	10 ± 0.578
E. coli GN9	12.6 ± 0.334	18.3 ± 0.334	10.6 ± 0.334	15.3 ± 0.334	12.6 ± 0.334	11.6 ± 0.334	13 ± 0.0	16 ± 0.0	11.3 ± 0.334	17.6 ± 0.334	15 ± 0.0
E. coli GN12	10.6 ± 0.667	19 ± 0.0	15 ± 0.0	10.3 ± 0.334	15.3 ± 0.334	14 ± 0.0	10.6 ± 0.334	19 ± 0.334	–	18.3 ± 0.334	10.6 ± 0.667
P. aeruginosa PA6	11.6 ± 0.334	20 ± 0.0	18.3 ± 0.334	12 ± 0.0	13 ± 0.0	15.3 ± 0.334	14 ± 0.0	20.3 ± 0.334	13 ± 0.0	17.6 ± 0.334	9.3 ± 0.334
E. coli GN2	–	18 ± 0.0	17.6 ± 0.334	10 ± 0.578	–	12.6 ± 0.334	–	21.3 ± 0.334	14 ± 0.0	20.3 ± 0.334	13 ± 0.0
K. pneumoniae KP5	–	13 ± 0.0	–	–	–	–	–	10.6 ± 0.334	–	10 ± 0.0	9.3 ± 0.334
E. coli EC16	–	12.3 ± 0.667	11 ± 0.0	–	11.6 ± 0.334	–	–	10 ± 0.578	10 ± 0.0	11.3 ± 0.334	11.3 ± 0.334
E. coli EC49	–	11.6 ± 0.334	–	9.3 ± 0.334	–	–	–	10 ± 0.334	10 ± 0.578	11.6 ± 0.334	11.6 ± 0.334
E. coli IB9	–	11.3 ± 0.334	–	11.6 ± 0.334	–	–	–	10.6 ± 0.334	–	12.3 ± 0.667	9.3 ± 0.334
E. coli GN13	11 ± 0.0	12.3 ± 0.667	12.3 ± 0.667	11.3 ± 0.334	–	11.6 ± 0.334	13.6 ± 0.334	11.6 ± 0.334	9.3 ± 0.334	10 ± 0.0	9.3 ± 0.334
A. baumannii AB-13773	–	11 ± 0.0	–	10 ± 0.0	–	–	–	9.3 ± 0.334	9.3 ± 0.334	15.3 ± 0.334	18 ± 0.0

– Indicates no inhibition; values are mean ± SEM of three replicates.
LAB = lactic acid bacteria; SEM = standard error of the mean.

database of type strains in manually curated and validly published prokaryotic names at the EzTaxon 2.1 server (https://www.ezbiocloud.net/resources/16s_download) (Chun *et al.* 2007). Molecular Evolutionary Genetics Analysis software (MEGA version 6.0) (http://megasoftware.net/) was used for phylogenetic analyses (Tamura *et al.* 2007). The sequences retrieved from database were aligned with the studied isolates using Clustal W inbuilt with MEGA 6.0. To construct the phylogenetic tree, neighbour-joining method was used with 500 bootstrap replications to assess nodal support in the tree.

3. Results

3.1. Isolation of LAB, antibacterial activity, characterization of inhibitory substances and bacteriocin production

A total of 84 isolates of LAB were obtained from the traditionally fermented products. Screening for the production of potential antimicrobial bacteriocin revealed 11 isolates (13.09%) inhibiting the growth of the indicator organisms and clinical isolates (Table 1; Figure 1).

The isolate LS5 showed the highest zone of inhibition (26 mm) against *E. faecalis*. *E. coli* was moderately inhibited by the bacteriocin with the highest zone of inhibition (23 mm) by the cell-free extract of the isolate LU11.

Of the 11 different MDR clinical pathogens tested, four bacteriocin-producing isolates (LS5, LS3, LU11 and LS6) exhibited antibacterial activity against all the clinical pathogens (Table 1; Figure 2). Among these, the isolates LS6 and LU11 showing high antibacterial activity against the tested indicator strains and clinical isolates were selected for further study.

Antibacterial activity of the cell-free supernatant showed no activity when treated with the proteolytic enzymes except pepsin. The inhibitory activity was not affected by the effect of catalase indicating that the growth inhibition was not due to hydrogen peroxide production (Table 2). The cell-free supernatant was stable to heat treatment at 60°C and 80°C for 60 min. However, heating at 100°C and 121°C reduced the activity by 30%–50% (Table 2). The antibacterial activity was also maintained stable over the pH range of 2–10 (Table 2).

3.2. Synergistic effect of LAB extract with antibiotics

The effect of bacteriocin extract from the isolates LU11 and LS6 alone and in combination with antibiotics against the MDR clinical pathogens *E. coli* (GN9, IB9, GN13), *K. pneumoniae* KP7 and *A. baumannii* AB13775 revealed different inhibitory effects (Table 3). The efficacy of polymyxin B and imipenem in combination with LU11 and LS6 extracts showed increased activity against all the strains of *E. coli* and *K. pneumoniae*. The bacteriocin extract of the isolate LU11 showed maximum increase in fold area of 1.086 with

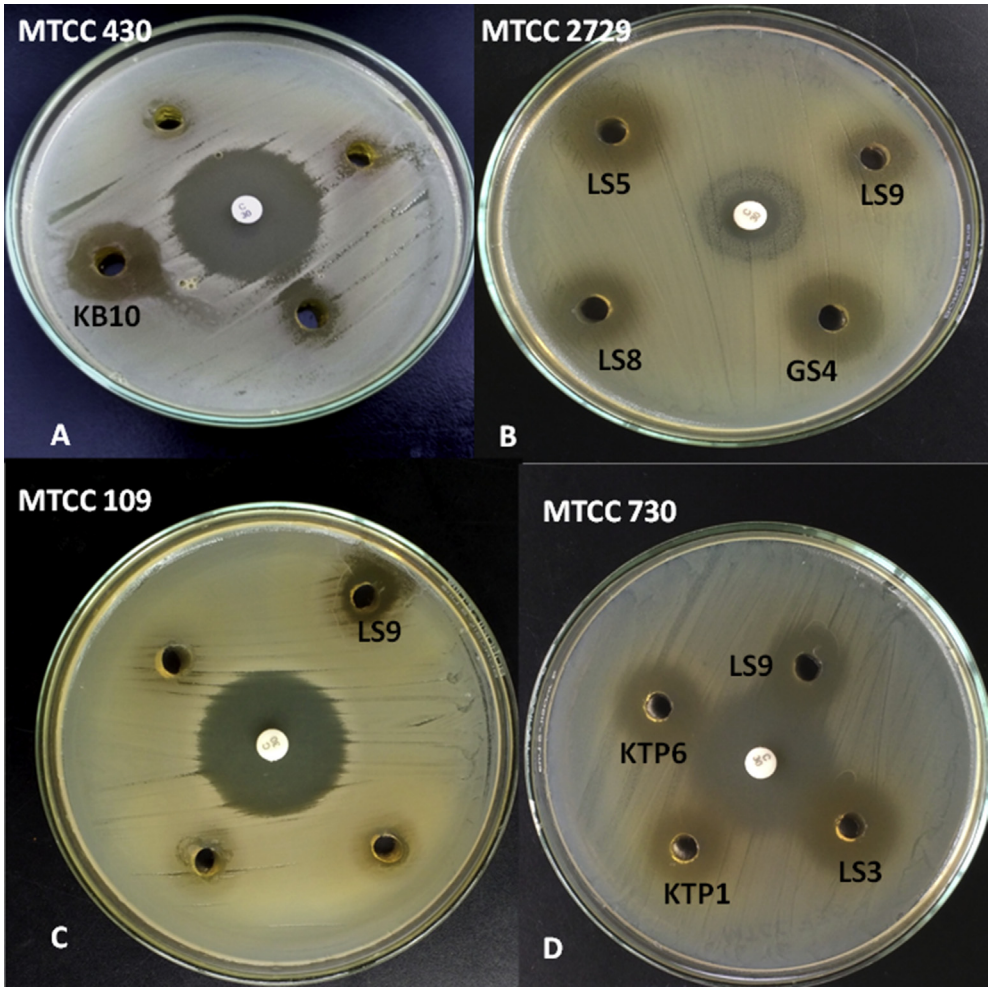

Figure 1. Agar well diffusion showing the bacteriocinogenic activities of representative isolates against **(A)** *Bacillus cereus* MTCC 430, **(B)** *Enterococcus faecalis* MTCC 2729, **(C)** *Klebsiella pneumoniae* MTCC 109 and **(D)** *Escherichia coli* MTCC 730. Each well contained the non–pH-adjusted cell-free extract of the isolates.

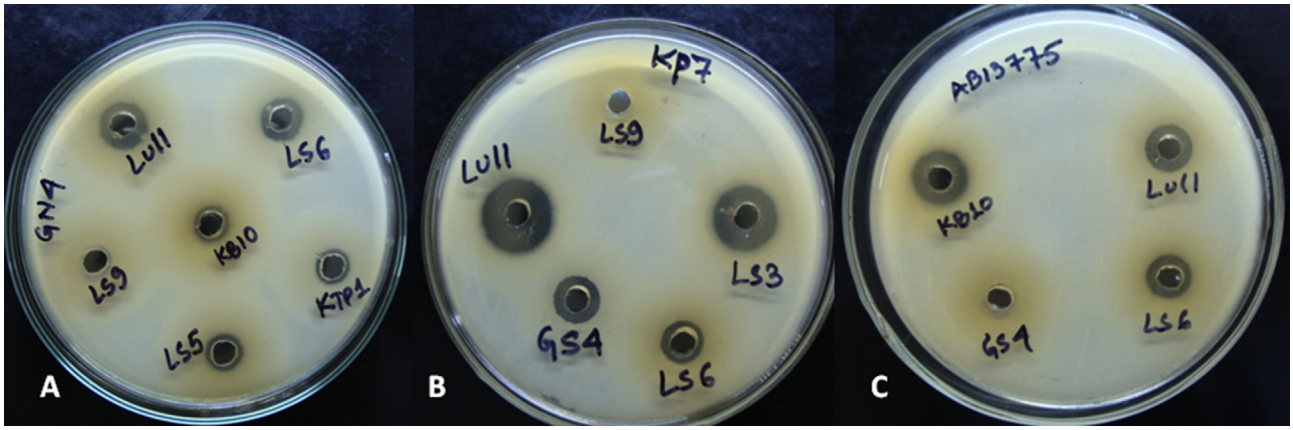

Figure 2. Agar well diffusion showing the bacteriocinogenic activities of representative isolates against clinical pathogens **(A)** *Escherichia coli*, **(B)** *Klebsiella pneumoniae* and **(C)** *Acinetobacter baumannii*.

Table 2. Physio-chemical characteristics of the bacteriocin produced by the LAB against *Escherichia coli* MTCC 730

Isolates	Control	Enzyme treatment					Effect of temperature				Effect of pH				
		Catalase	Pepsin	Proteinase K	Pronase E	Trypsin	60°C	80°C	100°C	121°C	2	4	6	8	10
LS9	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
LS5	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
GS4	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
KB10	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
KTP1	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
KTP5	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
KTP6	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
LS3	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
LS8	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
LU11	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++
LS6	+++	++	++	−	−	−	+++	+++	+	+	++	++	++	++	++

+++ Inhibitory zone > 10 mm; ++ inhibitory zone 7–10 mm; + inhibitory zone 5–7 mm; − no inhibitory zone.
LAB = lactic acid bacteria.

Table 3. Synergistic activity of bacteriocins alone and in combination with antibiotics

Mean of zone of inhibition (mm) produced by different antibiotics without/with bacteriocin													
	Test strains	PMB alone	PMB with bacteriocin extract	Increase in fold area	TGC alone	TGC with bacteriocin extract	Increase in fold area	IPM alone	IPM with bacteriocin extract	Increase in fold area	CTX alone	CTX with bacteriocin extract	Increase in fold area
Extract from isolate LU11	GN9	15.3 ± 0.334	16.16 ± 0.167	0.10 ± 0.003	13 ± 0.0	20.3 ± 0.334	0.57 ± 0.003	11 ± 0.0	13.3 ± 0.334	0.39 ± 0.0	−	10 ± 0.00	1 ± 0.00
	IB9	17 ± 0.0	20.3 ± 0.334	0.27 ± 0.003	25 ± 0.0	32.3 ± 0.334	0.63 ± 0.003	20 ± 0.0	22.3 ± 0.334	0.21 ± 0.0	−	28 ± 0.334	1 ± 0.007
	GN13	14 ± 0.0	17.3 ± 0.334	0.32 ± 0.003	21 ± 0.0	27.6 ± 0.334	0.65 ± 0.007	31 ± 0.0	31.6 ± 0.334	−	22 ± 0.0	24 ± 0.334	0.19 ± 0.003
	KP7	16 ± 0.0	20.3 ± 0.334	0.36 ± 0.003	18 ± 0.0	22.6 ± 0.334	0.49 ± 0.003	11 ± 0.0	13.6 ± 0.334	0.39 ± 0.007	−	10 ± 0.00	1 ± 0.00
	AB-13773	−	−	0.00 ± 0.003	18 ± 0.0	26 ± 0.0	1.086 ± 0.0	−	−	0.00	−	12 ± 0.334	1 ± 0.007
Extract from isolate LS6	GN9	15.3 ± 0.0	17.3 ± 0.334	0.14 ± 0.007	13 ± 0.0	21 ± 0.0	1.60 ± 0.0	11 ± 0.0	16.6 ± 0.334	1.11 ± 0.003	−	13.3 ± 0.334	1 ± 0.007
	IB9	17 ± 0.0	20.3 ± 0.334	0.38 ± 0.003	25 ± 0.0	31.3 ± 0.334	0.53 ± 0.0	20 ± 0.0	22.3 ± 0.334	0.21 ± 0.003	−	27 ± 0.00	1 ± 0.00
	GN13	14 ± 0.0	17.3 ± 0.334	0.47 ± 0.003	21 ± 0.0	27.6 ± 0.334	0.65 ± 0.0	31 ± 0.0	31.3 ± 0.334	−	22 ± 0.0	25	0.29
	KP7	16 ± 0.0	18.3 ± 0.334	0.26 ± 0.007	18 ± 0.0	24.3 ± 0.334	0.77 ± 0.003	11 ± 0.0	12.3 ± 0.334	0.19 ± 0.0	−	22 ± 0.00	1 ± 0.00
	AB-13773	−	−	−	18 ± 0.0	24.3 ± 0.334	0.77 ± 0.003	−	−	−	−	11 ± 0.00	1 ± 0.00

− Indicates no inhibition. Values are mean ± SEM of three replicates.
PMB = polymyxin B; TGC = tigecycline; IPM = imipenem; CTX = cefotaxime; SEM = standard error of the mean.

tigecycline against *A. baumannii* (Figure 3). Similarly, LS6 extract showed the highest activity in combination with tigecycline against *E. coli* GN9 with an increase in fold area of 1.60. Cefotaxime showed no inhibition in the growth of *E. coli* (GN9, IB9), *K. pneumoniae* (KP7) and *A. baumannii* (AB13775). However, the same antibiotic showed an increase in fold area when combined with the extracts of LU11 and LS6 (Table 3).

3.3. Minimum inhibitory concentration

The MIC value of the extracts correlated with the well-diffusion assay of bacteriocin activity (Table 4). The MIC of the bacteriocin extract of LU11 against *E. coli* MTCC 730 was 6.66 mg/mL, which was recorded as the lowest MIC value (Table 4). For the clinical isolates *E. coli* (GN9, IB9), *K. pneumoniae* (KP7) and *A. baumannii* (AB13775), the MIC was found to be 20.33, 26.66, 13.33 and 16.66 mg/mL, respectively, with the bacteriocin extract of LU11 (Table 4). The MIC of LS6 extract was 20 mg/mL against *E. coli* MTCC 730 and 22.66, 18, 10 and 33.33 mg/mL against *A. baumannii* AB13775, *K. pneumoniae* KP7 and *E. coli* (GN9, IB9), respectively. The bacteriocin extract from the isolate LU11 showed lower MIC values against *E. coli* MTCC 730 and the clinical pathogens *A. baumannii* AB13775 as well as *E. coli* IB9 in comparison with LS6 (Table 4).

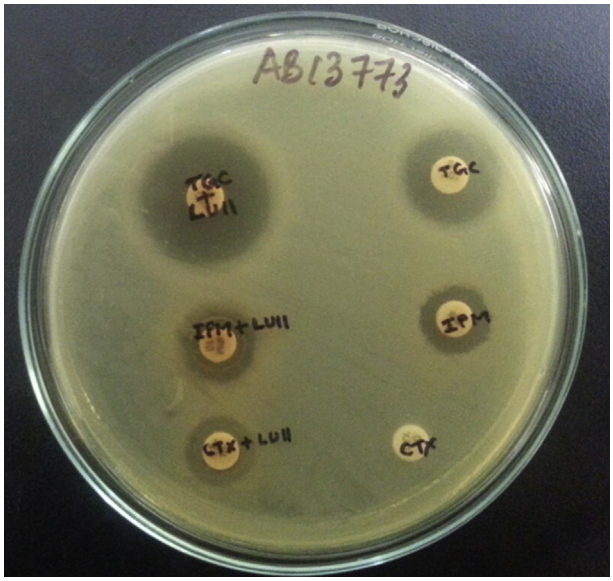

Figure 3. Synergistic effect of bacteriocins from the LAB isolate LU11 against clinical pathogens *A. baumannii*. The effect reflects the activity of bacteriocin alone and in combination with antibiotics tigecycline (TGC + LU11), imipenem (IMP + LU11) and cefotaxime (CTX + LU11). LAB = lactic acid bacteria.

3.4. Identification of potential bacteriocin-producing LAB

All the 11 potent bacteriocin-producing bacterial isolates were gram positive and catalase negative. Nine isolates were rod shaped and two were found to be cocci (Table 5). The isolates were characterised using 16S rRNA gene sequencing approach (Figure S3) and BLAST programme performed against the database at EzTaxon server to identify the nearest phylogenetic neighbours. Among the 11 bacteriocinogenic LAB isolates, *Lactobacillus* was the most predominant representing 72.7% of the isolates. *Pediococcus* comprised 18.18% of the isolates, whereas *Enterococcus* comprised 9.09% of the isolates. The bacterial isolates were found to be the closest homolog of *Lactobacillus plantarum* with a similarity of 99.80% (LS5, KB10, LS8, LS6), *Lactobacillus fermentum* with a similarity of 98.52% (GS4), *Pediococcus pentosaceus* with a similarity of 99.32% (LS9, LU11), *Lactobacillus casei* with a similarity of 98.54% (KTP1), *Lactobacillus brevis* with a similarity of 99.78% (KTP1, KTP5) and *Enterococcus* sp. with a similarity of 97.74% (LS3) (Table 5; Figure 4).

4. Discussion

Because of the broad spectrum of activity against bacteria and very low toxicity to humans, β-lactam group of antibiotics are widely used in the hospital settings for the treatment of human diseases. These antibiotics are rendered ineffective by bacteria producing β-lactamases, an enzyme capable of hydrolysing the β-lactam ring. Bacteriocins from LAB have huge potential to serve as food preservatives and also as next generation antibiotics, which can be used to target the MDR pathogens (Perez *et al.* 2014). Thus, the potential bacteriocin effective against these β-lactamase-producing organisms could serve as an alternative option for the treatment of hospitalized and community-based patients. In this study, LAB that are inherent to the traditionally fermented foods were isolated and explored for their potential to produce bacteriocins, which could inhibit the growth of β-lactamase-producing clinical isolates of gram-negative bacilli.

The study revealed 13% (11 of 84) of the isolates producing bacteriocin-like substances which could inhibit the growth of the indicator organisms. Sezer and Güven (2009) reported that only 35 LAB isolates exhibited antibacterial activity from a total of 12,700 LAB isolated from milk and meat products. Sharpe (2009) reported detection of 8.7% bacteriocin-producing strains among 92 LAB isolated from fresh cut vegetable products. In the present study, it was observed that all the eleven isolates had inhibited the growth of *E. coli, K. pneumoniae* and *B. cereus*, which corroborates to earlier reports (Mohankumar 2011; Rapsang *et al.* 2011, 2013). Difference in antimicrobial activity of the different isolates could be due to the different

Table 4. Minimum inhibitory concentration of bacteriocin extracts against the clinical isolates

LAB used for bacteriocin extract	MIC* (mg/mL) for the clinical isolates				
	Escherichia coli MTCC 730	*Acinetobacter baumannii* AB13775	*Klebsiella pneumoniae* KP7	*Escherichia coli* GN9	*Escherichia coli* IB9
Pediococcus pentosaceus LU11	6.66 ± 0.003	16.66 ± 0.003	13.33 ± 0.003	20.33 ± 0.003	26.66 ± 0.003
Lactobacillus plantarum LS6	20 ± 0.334	22.66 ± 0.003	18 ± 0.334	10 ± 0.334	33.33 ± 0.003

LAB = lactic acid bacteria; MIC = minimum inhibitory concentration; SEM = standard error of the mean.
* MIC values are mean ± SEM of three replicates..

Table 5. Biochemical parameters observed for the potent bacteriocin-producing LAB isolates

LAB isolates	Cell appearance	I	C	O	MR-VP	NR	U	CU	GH	Presumptive identification	Closest related microorganism* based on 16S rRNA sequences of the isolates	Similarity, %
LS9	Cocci	–	–	–	–	–	–	–	–	*Pediococcus* spp.	*Pediococcus pentosaceus*	99.369
LS5	Rods	–	–	–	–	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus plantarum*	99.796
GS4	Rods	–	–	–	+	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus fermentum*	98.521
KB10	Rods	–	–	–	–	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus plantarum*	99.836
KTP1	Rods	–	–	–	–	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus casei*	98.545
KTP5	Rods	–	–	–	–	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus brevis*	99.736
KTP6	Rods	–	–	–	–	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus brevis*	99.781
LS3	Rods	–	–	–	–	–	–	–	–	*Enterococcus* spp.	*Enterococcus* sp.	97.740
LS8	Rods	–	–	–	–	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus plantarum*	99.801
LU11	Cocci	–	–	–	+	–	–	–	–	*Pediococcus* spp.	*Pediococcus pentosaceus*	99.329
LS6	Rods	–	–	–	–	–	–	–	–	*Lactobacillus* spp.	*Lactobacillus plantarum*	98.796

+ = positive; – = negative; C = catalase test; CU = citrate utilization; GH = gelatin hydrolysis; I = indole production; LAB = lactic acid bacteria; NR = nitrate reduction; O = oxidase test; U = urease production, MR-VP = Methyl Red, Voges-Proskauer biochemical test.
* Validly published species in EzTaxon server..

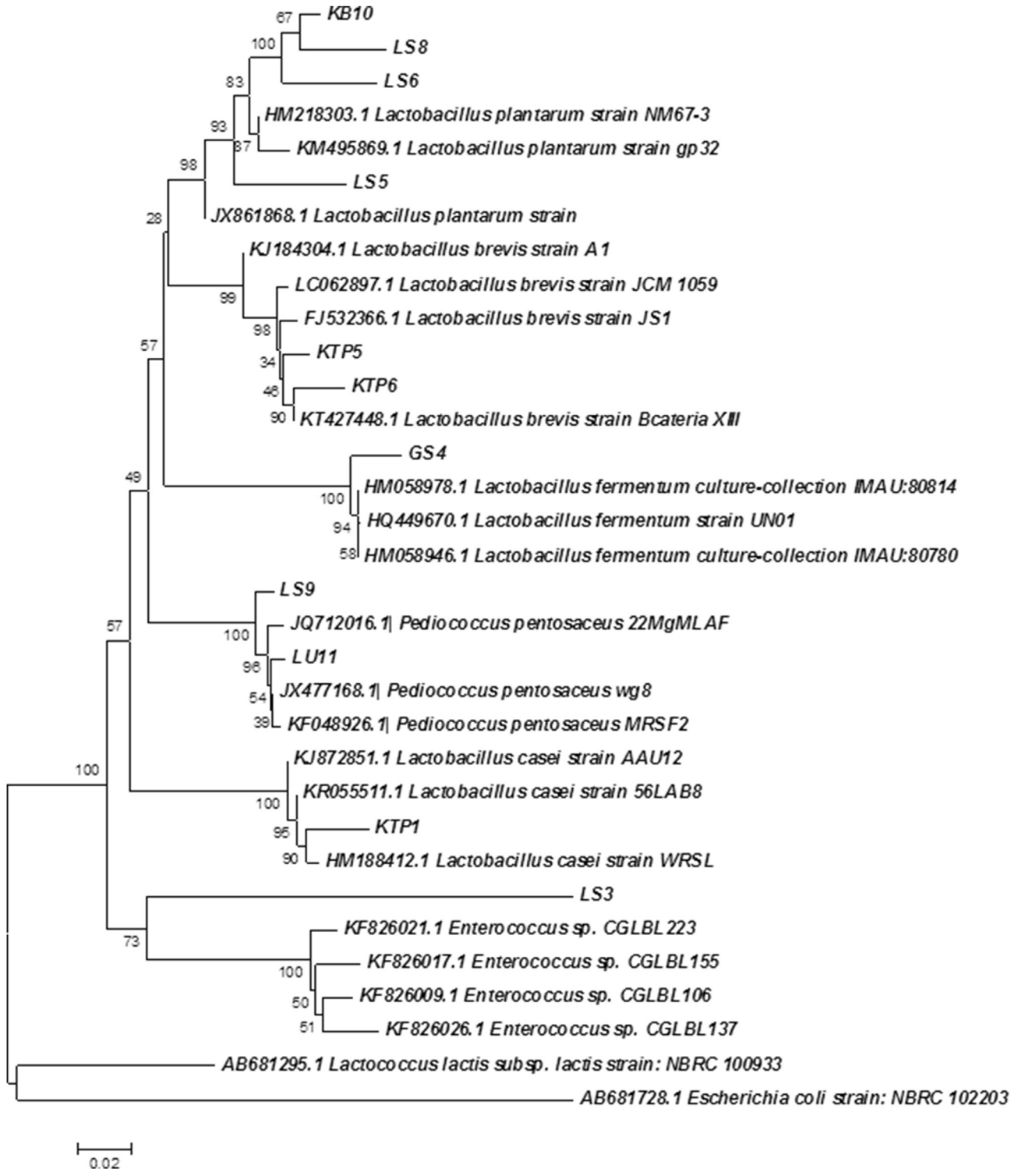

Figure 4. Neighbour-joining tree showing the phylogenetic position of isolated strains based on 16S rDNA gene sequence. Evolutionary distance was calculated using Kimura 2-parameter model. Bootstrap values based on 500 replicons are shown.

pathway for the production of lactic acid by homofermentative and heterofermentative lactobacilli (Tejero Sarinena *et al.* 2012).

Physio-chemical characterization of the cell-free supernatant was carried out by treating the bacteriocin produced by the LAB with different enzymes and also by subjecting them to different temperature and pH ranges. Treatment of the bacteriocin with various proteolytic enzymes (trypsin, proteinase K, pronase E) resulted in the loss of the antibacterial activity. This indicated the proteinaceous nature of the compounds. Same set of proteolytic enzymes also inactivated nisin (Hurst 1981) and similar results have also been reported by Gajić *et al.* (1999). Bacteriocins are relatively heat stable molecules. The activity of the cell-

free supernatant was retained at various pH ranges and was not affected by heat treatment at 80°C for 60 min. The work carried out by Lade *et al.* (2006) also reported that bacteriocin isolates were stable at 100°C for 10 min and bacteriocin of *Lactobacillus lactis* was stable in acidic condition. Sarika *et al.* (2010) also reported heat and pH stability of bacteriocin Lactobacillus *rhamnosus*.

Because of their potential use as antimicrobial agents for improving the safety of food products, there is an increasing interest in the research of antimicrobial peptides (bacteriocins and bacteriocin-like compounds) produced by LAB (Yildirim *et al.* 1999). Apart from its inhibition spectrum, technological properties like heat and wide range of pH stability provide the bacteriocin with an application potential as a biopreservative (Abo-Amer, 2007). Although biochemically and genetically many bacteriocins have been characterized, several aspects of these compounds are still unknown (Cleveland *et al.* 2001), this explains why till date nisin, approved by the Food and Drug Administration, is the only purified bacteriocin widely used as a food preservative.

The present study also explored the antibacterial spectrum of the LAB isolates against MDR bacterial species. There are reports on the efficacy of the commercially available bacteriocin, nisin against pathogenic strains of MDR *S. aureus*, pneumococci and MRSA (Severina *et al.* 1998). The active supernatants of the potential isolates *L. plantarum* LS6 and *P. pentosaceus* LU11 showed good inhibitory effects against the bacteria harbouring β-lactamases. Bacteriocins from these isolates had a broader host range against the clinical isolates. Among the nosocomial strains used in the study, *K. pneumoniae* and *A. baumannii* have proven to be sensitive targets of the bacteriocin extracted from *L. plantarum* LS6 and *P. pentosaceus* LU11. Bacteriocin extract from these isolates also showed good inhibition activity and MIC against isolates of *E. coli*, producing extended-spectrum β-lactamases and metallo-β-lactamases. The MIC value of *P. pentosaceus* crude extract against the gram-negative nosocomial isolate *K. pneumoniae* and *A. baumannii* was recorded to be lower than the MIC value obtained for the extract of *L. plantarum*. Jang *et al.* (2015) studied the activity of *P. pentosaceus* T1 against *Listeria monocytogenes* and reported that the MIC of this pediocin isoform (20 mg/mL) is lower than that of nisin.

L. plantarum strains produce a broad range of bacteriocins such as ST28MS, ST26MS, bacST202Ch, bacST216Ch, ST71KS, plantaricin B, D, K, MG, JKZJ008, etc (Dinev *et al.* 2017). According to previous studies, it is reported that *L. plantarum* is active against many Gram-negative pathogens, food spoilage microorganisms and various MDR (Dinev *et al.* 2017). Zang *et al.* (2016) reported that *L. plantarum* ZDY 2013 significantly inhibited the adhesion of enterotoxin-producing and pathogenic strains of *B. cereus* on intentional epithelial cells by inhibition, competition and displacement. The antimicrobial activity of LAB and *L. plantarum* can be explained by three mechanisms: the production of bacteriocins; the yield of organic acids, ethanol, carbon dioxide and hydrogen peroxide; and the competition of nutrients (Magnusson *et al.* 2003). Our results showed that the strain of *L. plantarum* has potential to produce antibacterial substances, which can be viable alternative to antibiotics to treat the pandrug-resistant organisms. Kumar *et al.* (2016) also concluded that *L. plantarum* strains which are effective against a variety of bacterial pathogens including MRSA and MDR enteroaggregative *E. coli* can serve as alternative therapeutic agents against the corresponding infections in humans and animals.

Similar to *L. plantarum, Pediococcus* cultures are evident as protective cultures against common food spoilage bacteria and pathogens. *P. pentosaceus* is one of the main species used in pediocin production, fermentation processes as starter culture and probiotic supplements for humans and animals (Porto *et al.* 2017). Recently, several studies have demonstrated pediocin production from different *P. pentosaceus* strains such as *P. pentosaceus* CB4, R38, OZF, NDCD273 (Devi and Halami 2011; Osmanagaoglu *et al.* 2013; Simha *et al.* 2012). In a study performed by Yin *et al.* 2003, it was reported that pediocin from *P. pentosaceus* inhibited the growth of various Gram-negative microorganisms.

Based on the varied investigations, *P. pentosaceus* and *L. plantarum* can be considered as important LAB species and the purified form of their bacteriocins can be explored as potent antibacterial candidate for the treatment of nosocomial infections.

One of the greatest concerns that mankind is facing today is the rising problem of MDR pathogens and the rapidly decreasing spectrum of antibiotics to control these pathogens. In the present study, it was observed that the bacteriocin from the LAB alone and in combination with polymyxin B, tigecycline, imipenem and cefotaxime had an inhibitory activity on MDR strains of bacteria. Although these antimicrobials cannot replace the widely used antibiotics, they may serve as valuable antibiotic complements. Traditionally fermented products harbouring a range of probiotics LAB is a rich gene pool of organisms providing such complements. To take advantage of these new antimicrobials in synergistic combination therapy, it will be essential to determine the best possible ratio and dosing treatment and to study the mechanism of their activity exploring metabolomic technologies.

Author contributions

All authors contributed equally to the work.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.hjb.2017.08.008.