



## A comparative profile of bactericidal action of a partially purified bacteriocin from lactic acid bacteria with antibiotics

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### ABSTRACT

**Aims:** The menace of antibiotic resistance has led to the search for alternatives, which in turn has diverted the attention to bacteriocins, antimicrobial peptides (AMPs) produced by bacteria for their bactericidal properties. The aim of our study was to isolate and partially purify bacteriocin from lactic acid bacteria (LAB) and comparing its antimicrobial activity with antibiotics.

**Methodology and results:** Among 38 LAB screened using agar spot assay, LAB 28D1 showed the highest antimicrobial activity against the test bacterial strains. The proteinaceous nature of the antimicrobial compound extracted from LAB 28D1 was confirmed by its inactivation after treatment with proteolytic enzymes. The crude bacteriocin was found to be stable over a wide range of temperatures (60-100 °C) and pH (4-9). The bacteriocin was partially purified by ammonium sulfate precipitation (ASP) and the activity units were 204,800 AU/mL. The total protein and the specific activity of partially purified bacteriocin were found to be 24.585 mg and 124,954.24 AU/mg respectively. The molecular weight of partially purified bacteriocin was determined to be 8.5 kDa approximately. The efficacy of the partially purified bacteriocin against indicator bacterial strains was compared with antibiotics by the disc diffusion method and minimum inhibitory concentration (MIC). According to our study, the hospital waste isolate *Enterococcus* spp. was found to be multidrug-resistant (MDR) but sensitive to bacteriocin from LAB (MIC 0.06 ± 0 µg/mL).

**Conclusion, significance and impact of the study:** Bacteriocin from LAB has potential in combating MDR enterococcal infections.

**Keywords:** Bacteriocin, lactic acid bacteria, partial purification, antibiotics, multi-drug resistance

### INTRODUCTION

Bacteriocins are peptide molecules synthesized by microorganisms mainly bacteria, but also some species of archaea to competitively inhibit the rival organisms. (Chikindas *et al.*, 2017; Tashakor *et al.*, 2017; Lopetuso *et al.*, 2019). Bacteriocins derived from lactic acid bacteria (LAB) are very small (>10 kDa) ribosomal peptides which can inhibit wide range of bacteria (Drago *et al.*, 1997). LAB are commensal probiotic bacteria forming normal gut flora with a positive effect on host wellbeing. These probiotic organisms play a powerful role in eliminating contaminated food and water-based infections like diarrhoea and food poisoning (Rapsang *et al.*, 2013; Martha *et al.*, 2001). The commensal bacteria produce bacteriocins, which display bactericidal activity against some groups of bacteria (Rapsang *et al.*, 2013). The bacteriocin mechanism action involves degrading the target bacterial cell wall or cell membrane by pore form or by any nuclear activity like DNase and RNase. It disrupts

the outer surface coat of the affected bacteria. Due to the loss of water and electrolytes, it changes the membrane potential of the bacteria and causes the bacteria fail to survive (da Silva Sabo *et al.*, 2014; Sharma *et al.*, 2018; Perez *et al.*, 2018).

The conventional antibiotics are the mainstay for treating infectious disease. But injudicious, excessive and incorrect use of conventional antibiotics has led to the phenomenon of antibiotic resistance. As a result, many traditional antibiotics show a lack of potency when it comes to killing pathogenic organisms. Therefore, there is a need to improve the processes associated with antibiotic consumption and also to find newer antibiotics and alternative therapeutic agents to overcome antibiotic resistance problem. Antimicrobial peptides are one among options being studied (Montalbán-López *et al.*, 2011; Naghmouchi *et al.*, 2013).

A large part of the population is affected by disease caused by antibiotic resistant bacteria and this number is increasing every year. The critical issue of growing

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antibiotic resistance is a major threat to human well-being. The scarcity in terms of antibiotics required to tackle the MDR *Staphylococcus aureus* strains is a matter of grave concern (Santos *et al.*, 2017; Mathur *et al.*, 2018). Bacteriocins are under the spotlight because of their therapeutic potential with the added benefits of low side effects and being newer agents, the development of resistance would be also slower (Cotter *et al.*, 2013).

Bacteriocins from LAB have already proven to be useful in the food industry where they have helped increase the shelf life of foodstuff (Lianou *et al.*, 2017; Mills *et al.*, 2017; Anacarso *et al.*, 2017; Hammami *et al.*, 2019; Papagianni and Anastasiadou, 2019; Costa *et al.*, 2019). It has been reported lactobacillus is responsible for the production of most of the bacteriocins. These proteins are thermally stable type II bacteriocins (Corr *et al.*, 2007) and play an important role in the initiation of cell membrane rupture, which leads to leakage of cell organelles and cell death (Garneau *et al.*, 2002).

The effective action of bacteriocins isolated from *Lactobacillus* of common clinical pathogens has been reported *in vitro* (Todorov and Dicks, 2005; Svetoch *et al.*, 2011). But lesser study is available concerning the comparative study of the antibacterial action of bacteriocin and antibiotics (Gholami *et al.*, 2018). Thus, the present study deals with isolation and partial purification of bacteriocin from LAB and comparing its efficacy with antibiotic against different indicator bacterial strains.

## MATERIALS AND METHODS

### Isolation of lactic acid bacteria (LAB)

In the present study, dairy food (home-made curd) samples were collected from different areas of Karnal and Delhi, India. Samples were diluted serially till  $10^{-6}$  dilution. LAB cultures were isolated in De Man, Rogosa and Sharpe (MRS) agar media (De Man *et al.*, 1960). MRS agar media (HiMedia Laboratories Pvt. Ltd., Mumbai, India) with bromocresol purple was applied to identify LAB strains.

### Tested bacterial strains and growth media

The tested bacterial strains *Enterococcus faecalis* NCDC 114, *Bacillus subtilis* NCDC 70, *Sarcina lutea* NCDC 112 and *Micrococcus luteus* NCDC 174 were procured from National Collections of Dairy Cultures (NCDC), Karnal, India. The cultures were maintained in nutrient agar plates (HiMedia Laboratories Pvt. Ltd., Mumbai, India).

### Determination of the antibacterial activity of LAB by agar spot assay

LAB strains were screened for their antibacterial action against tested bacterial strains by agar-spot assay (Fleming *et al.*, 1975). LAB cultures and indicator strains were activated in nutrient broth media by three successive transfers with intervals of 18 h at 37 °C for spot-inoculation on agar plates. The surface of the tryptone

glucose extract (TGE) agar plates were spot inoculated with LAB strains (5 µL) and incubated overnight at 37 °C. After incubation, the plates were overlaid with 7 mL of TGE soft agar (0.8% agar) medium seeded with 30 µL of test bacterial strains and again incubated overnight at 37 °C. A halo zone greater than 2 mm extending from the border of the bacterial spot was recorded as a positive result.

### Agar well diffusion assay

The cell-free culture supernatants (CFCS) were obtained by centrifuging (Hettich Mikro 22R Refrigerated Centrifuge, UK) the screened LAB cultures at 10,000 rpm for 15 min at 4 °C. The CFCS was treated at 90 °C for 7 min to eliminate any live bacteria. The pH of CFCS was set to 6.5 with the help of 1 N NaOH. TGE agar media/ TGE soft agar media (HiMedia Laboratories Pvt. Ltd., Mumbai, India) was applied to observe the antibacterial activity of LAB (Biswas *et al.*, 1991). The test strains were cultured in nutrient broth for 18 h at 37 °C. Fifty microlitres of broth culture were added to the sterile fresh nutrient broth and kept for 3 h at 37 °C to obtain an active culture. Melted TGE soft agar containing 30 µL of active culture was poured onto TGE agar plates. In this method, 6 mm wells were made using a sterile borer inside the TGE agar plates (inoculated with test bacterial strains) followed by sealing of wells surfaces with melted TGE agar. The wells were loaded with 100 µL of CFCS and incubated overnight to observe the zone of inhibition (Barefoot and Klaenhammer, 1983).

### Determination of activity unit of crude bacteriocin by spot-on-lawn assay

To determine the activity units of crude bacteriocin, the CFCS was serially diluted and spotted 5 µL of each dilution on the TGE agar plate seeded with test bacterial strain. The plate was incubated overnight to form clear zone of inhibition. The activity unit of crude bacteriocin was measured by the following formula:

Activity units per mL (AU/mL) =  
 $200 \times \text{Reciprocal of the highest dilution that gives a clear zone (Uhlman et al., 1992)}$

Total activity units (AU) of CFCS =  
Total volume (mL) of CFCS  $\times$  Activity units per mL

### Identification of bacteriocin producing LAB

The genus of the bacteriocin producing LAB was determined based on morphology and biochemical characteristics (Ahmed *et al.*, 2004).

## Stability of crude bacteriocin to enzyme, temperature, and pH

### Enzyme treatment

The crude bacteriocin was treated with protease, pepsin, trypsin,  $\alpha$ -chymotrypsin, catalase, and lipase. All the enzymes were procured from Sigma-Aldrich Corporation, USA and suspended in phosphate buffer (pH 7). Except for pepsin, which was suspended in 0.02 N hydrochloric acid (pH 3). The residual activity of enzyme treated crude bacteriocin was estimated by spot-on-lawn assay.

### Temperature stability

The stability of crude bacteriocin was analysed over a range of temperatures starting from 60 °C to 121 °C (autoclave temperature). After the treatment, residual activity of crude bacteriocin was estimated by spot-on-lawn assay.

### pH stability

The stability of crude bacteriocin was analyzed over a range of pH 1-10. The buffers used were: HCl-KCl buffer for pH 1 and 2, glycine HCl buffer for pH 3, acetate buffer for pH 4 and 5, phosphate buffer for pH 6 and 7, Tris-HCl buffer for pH 8 and 9 and glycine-NaOH buffer for pH 10. After pH treatment, residual activity of crude bacteriocin was measured by spot-on-lawn assay.

## Partial purification of crude bacteriocin by ammonium sulfate precipitation (ASP)

To obtain precipitation of protein, ammonium sulfate salt was slowly added to 200 mL of CFCS (crude bacteriocin) at 4 °C to reach saturation levels of 60% with continuous stirring using a magnetic stirrer. The precipitated mixture was centrifuged at 10,000 rpm for 20 min at 4 °C. Ammonium sulfate concentrations were raised to get 70% and 80% level of saturation. The precipitate was collected every time with the help of a spatula and was dissolved in 15 mL of 0.5 M phosphate buffer. The salt in the solution was eliminated by dialysis and filtered through a 0.22  $\mu$ m membrane. The activity unit of partially purified bacteriocin was measured. The protein content of crude bacteriocin and partially purified bacteriocin was estimated as described by Lowry *et al.* (1951) using bovine serum albumin as standard. Specific activity was determined as the ratio of total activity units of the bacteriocin (AU) and the total protein (mg).

## Determination of molecular weight by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of bacteriocin was determined using SDS-PAGE as illustrated by Schagger and Jagow (1987). The *in situ* activity of bacteriocin was also observed. The SDS-PAGE was carried out at 50 volts and continued until

the tracking dye made its way to the required endpoint. Then, the gel was cut into half. The first half of the stained gel consists of the sample and protein molecular weight marker stained with 0.1% Coomassie Brilliant Blue R-250. The second half of the unstained gel with bacteriocin samples was washed by sterile double distilled water for 4 h. The water used for washing was replaced for every 40 min with the freshwater for efficient cleaning of the gel. The gel was overlaid with 10 mL of TGE soft 0.8% agar containing 50  $\mu$ L of *E. faecalis* NCDC 114 (fresh culture). The gel with TGE soft agar was incubated at 37 °C for 18 h to observe the zone of inhibition. The position of protein molecular weight marker was compared with the midpoint of the zone of inhibition to get an approximate value of bacteriocin molecular weight.

## Preparation of bacteriocin disc

Bacteriocin discs were prepared with Whatman filter paper No. 1 (Sigma-Aldrich, USA) with the help of a punching machine of 6 mm in diameter. The discs were sterilized by autoclaving. Partially purified bacteriocin stock solution was prepared which was subsequently diluted (100x) to obtain a working solution. A 20  $\mu$ L of the working solution was added to each disc and subsequently air-dried.

## Bacteriocin and antibiotic susceptibility test

The indicator strains *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923, *E. coli* ATCC 35218, *E. faecalis* ATCC 29212 were procured from National Chemical Laboratory, Pune, India. On the other hand, hospital waste isolated strain *Enterococcus* spp. and *Bacillus* spp. were collected from Fortis Hospital, Vasant Kunj, New Delhi, India. Nutrient agar media was used to maintain the cultures. Antibiotic or bacteriocin sensitivity test was performed by disc diffusion method as per Clinical and Laboratory Standards Institute (CLSI), 2015. The surface of Muller Hinton agar (MHA) plates were inoculated with tested bacterial suspension of 0.5 McFarland turbidity standards. The antibiotic discs/bacteriocin disc used were gentamicin (30  $\mu$ g), amikacin (30  $\mu$ g), ampicillin (10  $\mu$ g), bacitracin (10 U), ciprofloxacin (5  $\mu$ g), gemifloxacin (5  $\mu$ g), tetracycline (15  $\mu$ g) and bacteriocin (3.2  $\mu$ g). All discs were aseptically placed on the surface of indicator bacteria seeded media plates and incubated for 18 h at 37 °C. After incubation, the zone of inhibition was measured. The results were finalized as sensitive, intermediate and resistant as per the particular antibiotics references (CLSI, 2015).

## Determination of minimum inhibitory concentration (MIC)

The stock solutions of all the seven antibiotics were collected from HiMedia Laboratories Pvt. Ltd., Mumbai, India. All the antibiotic stock solutions were stored at -20 °C. The range of dilution of different antibiotics is shown in Table 1. From the initial stock solution (10,000 mg/L), dilutions of all the antibiotics were made in a concentration

of 1,000 and 100 µg/L. In the case of bacteriocin, 100× dilution was prepared from the partially purified bacteriocin stock solution. The microdilution was done in a 96 well plate format (Emery Pharma, USA) as per CLSI guidelines, 2018. All the indicator bacterial strains were prepared for inoculum of 0.5 McFarland turbidity standard verifying the optical density (OD) by using a spectrophotometer (Shimadzu UV-1800). The bacterial inoculum was added to the antimicrobial agent along with the growth media and incubated for a period of 18-20 h at 37 °C. To visualize the MIC value, a mirror reader was used to examine each well for visible bacterial growth. The lowest concentration of antibiotic/bacteriocin that showed no visible growth was the MIC of a particular antibiotic/bacteriocin.

**Table 1:** Target MIC ranges (µg/mL) of antibiotics and bacteriocin for indicator bacterial strains.

Antibiotics*	Concentration range (µg/mL)
Amikacin	0.06-128
Gentamicin	0.03-64
Tetracycline	0.06-256
Ampicillin	0.15-256
Gemifloxacin	0.002-16
Bacitracin	0.2-256
Ciprofloxacin	0.15-32
Bacteriocin from LAB	0.008-16

\*Source: British Society for Antimicrobial Chemotherapy, 1991

## RESULTS

### Screening for crude bacteriocin production from LAB

The agar spot method was used for the primary screening of LAB for their antimicrobial activity. Thirty-eight LAB strains were found to be active against tested bacterial strains. Figure 1 shows the screening of LAB against *E. faecalis* NCDC 114 by the agar spot method. The CFCS of 38 LAB strains were observed for their crude bacteriocin production using tested bacterial strains by agar well diffusion assay (Table 2). Among 38 LAB strains, the crude bacteriocin extracted from 28D1 showed the highest antimicrobial activity against all the test bacterial strains. Out of 4 tested bacterial strains, *E. faecalis* NCDC 114 was found to be most sensitive to crude bacteriocin of 28D1 (Figure 2).

### Calculation of activity units of crude bacteriocin

The activity unit of crude bacteriocin 28D1 was calculated as 12,800 AU/mL against *E. faecalis* NCDC114 (Figure 3).

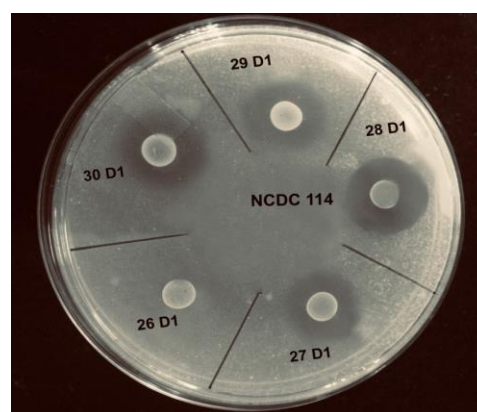
### Identification of bacteriocin producing isolate 28D1

LAB 28D1 was identified as Gram-positive bacilli. The growth was seen at 45 °C but no growth was observed at 15 °C. It was catalase-negative, able to hydrolyze aesculin but showed a negative result in arginine hydrolysis. The strain showed no gas production from glucose. These

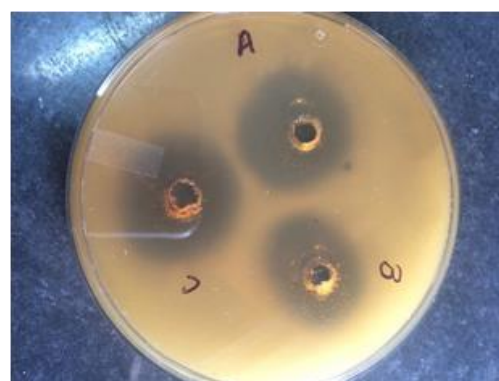
characteristics proved that 28D1 belonged to the genus *Lactobacillus* group.

### Stability of crude bacteriocin to enzyme, temperature, and pH

Table 3 shows the effect of type of enzyme, temperature and pH to the residual crude bacteriocin activity. Proteolytic enzymes like protease, trypsin, chymotrypsin, disrupted the bacteriocin structure and function because of their peptide nature and resulted in a loss of activity. On the other hand, non-proteolytic enzymes like lipase and catalase did not affect the crude bacteriocin activity. The residual activity of bacteriocin was analyzed over an extended range of temperature and pH. The temperature ranged between 60 °C to 121 °C and pH varied between 1 and 10. Table 3 shows that the bacteriocin activity was unaffected between 60-80 °C, but progressively diminished as the temperature was increased. Ninety-



**Figure 1:** Screening of LAB isolates for antimicrobial activity against *E. faecalis* NCDC 114 by agar spot assay. Zone of inhibition (26D1 = 0 mm, 27D1 = 22 mm, 28D1 = 26 mm, 29D1 = 24 mm, 30D1 = 25 mm).

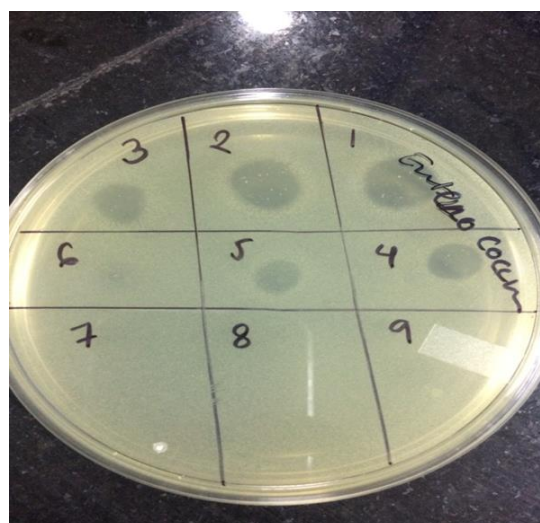


**Figure 2:** Screening for crude bacteriocin production from LAB isolates against *E. faecalis* NCDC 114 by agar well diffusion assay. A, 30D1; B, 3D1; C, 28D1. The zone of inhibition of 30D1 = 29 mm; 3D1 = 26 mm; 28D1 = 32 mm.

three percent residual activity was observed at 90 °C and reduced to 64% at 100 °C for 60 min. The activity was found to be negligible after autoclaving. The bacteriocin activity was negligible at the highly acidic pH (1-3) and then progressively increased to 100% activity at pH 6 and 7. The activity again declined in alkaline pH being 76% at pH 8 and being absent at pH 10.

**Table 2:** Screening for crude bacteriocin production from LAB isolates against tested bacterial strains using agar well diffusion assay.

CFCS of LAB Isolates	Zone of inhibition (mm)			
	<i>B. subtilis</i> NCDC 70	<i>S. lutea</i> NCDC 112	<i>E. faecalis</i> NCDC 114	<i>M. luteus</i> NCDC 174
1D1	14	-	15	-
1D2	-	-	-	-
1D4	-	-	12	-
2D2	-	14	-	-
3D1	9	18	26	10
4D4	-	-	8	-
5D1	13	-	12	12
6D1	-	14	18	23
6D4	14	-	-	-
7D1	-	-	-	-
8D1	15	-	14	8
14D1	9	16	21	-
15D1	-	-	-	-
19D1	11	-	17	-
20D2	8	-	12	13
23D1	-	-	-	-
24D2	-	-	-	-
27D1	-	-	24	-
28D1	14	16	32	20
29D1	10	-	25	13
30D1	6	-	29	8
31D2	-	11	-	14
34D2	6	-	10	-
36D1	-	09	-	-
36D4	-	-	12	-
37D3	2	-	6	-
38D2	-	8	-	12
40D1	-	-	16	-
41D3	-	-	-	-
43D1	-	-	14	-
43D2	5	-	-	-
44D2	-	-	8	-
46D2	12	-	6	-
47D2	-	-	-	-
48D1	-	-	-	-
49D1	-	11	-	-
49D3	-	-	12	8
50D1	4	-	6	-



**Figure 3:** Determination of activity units of crude bacteriocin against *E. faecalis* NCDC 114 by the spot-on-lawn assay. Here, 6 is the highest dilution that gives a clear zone. The activity unit of crude bacteriocin 28D1 =  $200 \times 2^6 = 12,800$  AU/mL.

**Table 3:** Effect of enzymes, temperature, and pH on crude bacteriocin.

Factor	Treatment	Residual bacteriocin activity (%)*
Enzymes	Protease	Negligible
	Trypsin	Negligible
	Chymotrypsin	Negligible
	Pepsin	Negligible
	Catalase	100
	Lipase	100
Temperature	60 °C/10 min	100
	80 °C/10 min	100
	90 °C/10 min	93
	100 °C/10 min	88
	100 °C/30 min	76
	100 °C/60 min	64
pH	121 °C/15 min	Negligible
	1	Negligible
	2	Negligible
	3	Negligible
	4	62
	5	98
	6	100
	7	100
	8	76
	9	42
10	Negligible	

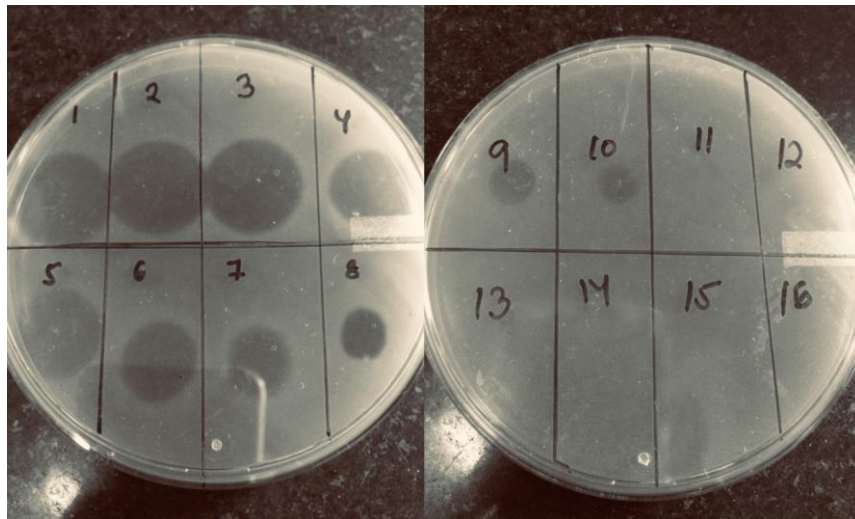
\*Residual bacteriocin activity (%) = Bacteriocin activity in the treated sample / bacteriocin activity in the untreated sample x 100%.



### Partial purification of crude bacteriocin

The total activity and specific activity of crude bacteriocin were determined to be 2,560,000 AU and 7578.44 AU/mg respectively. The crude bacteriocin was concentrated by precipitation with 80% saturation of ammonium sulphate salt. After dialysis, the antimicrobial activity of partially purified bacteriocin was found to be 204,800 AU/mL against *E. faecalis* NCDC 114 (Figure 4). The total activity

unit of partially purified bacteriocin was calculated 3,072,000 AU, 16.4-fold of purification and 120% overall yield compared to crude bacteriocin (Table 4). The estimated molecular weight of partially purified bacteriocin by SDS-PAGE was found to be approximately 8.5 kDa size while comparing with both the centre of the inhibitory zone on the gel (*in situ* activity of bacteriocin) and the molecular weight marker (Figure 5).



**Figure 4:** Determination of activity units of partially purified bacteriocin against *E. faecalis* NCDC 114 by the spot-on-lawn assay. The activity unit of partially purified bacteriocin =  $200 \times 2^{10} = 204800$  AU/mL.

**Table 4:** Comparison of crude bacteriocin and partially purified bacteriocin.

Sample	Total volume (mL)	Total activity units (AU)	Total protein (mg)	Specific activity <sup>a</sup> (AU/mg)	Purification fold <sup>b</sup>	Overall yield <sup>c</sup> (%)
Crude bacteriocin	200	2,560,000	337.8	7,578.44	1	100
Partially purified bacteriocin	15	3,072,000	24.585	124,954.24	16.4	120

<sup>a</sup>Specific activity = Total activity units / total protein.

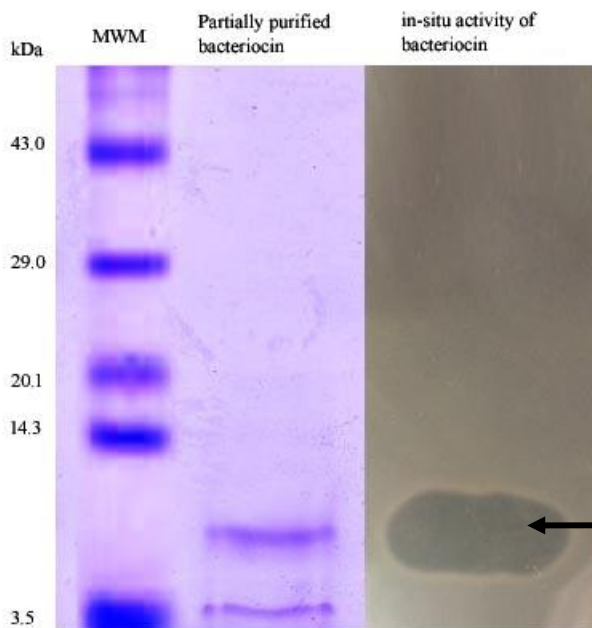
<sup>b</sup>Purification fold = Specific activity of partially purified bacteriocin / specific activity of crude bacteriocin.

<sup>c</sup>Overall yield = Total activity units of partially purified bacteriocin / total activity units of crude bacteriocin × 100

### Comparison of the antibacterial activity of bacteriocin (partially purified) with antibiotics by disc diffusion method

In Figure 6, the antibacterial activity of bacteriocin and antibiotics against indicator bacterial strains using the disc diffusion assay is represented by a bar graph. Inhibition zone diameters of all antibiotics were interpreted according to CLSI guidelines, 2015. The bacteriocin was least effective against *P. aeruginosa* ATCC 27853 than other antibiotics except, ampicillin. *P. aeruginosa* ATCC 27853 was resistant to ampicillin. Besides, the antimicrobial activity of bacteriocin against *E. coli* ATCC 25922 was higher than tetracycline, bacitracin, and

ampicillin, almost similar to gentamicin and amikacin but much lower than ciprofloxacin and gemifloxacin. Again, the antimicrobial activity of bacteriocin against *S. aureus* ATCC 25923 was almost similar to that of gentamicin, gemifloxacin and tetracycline, except for ampicillin, where it was higher than bacteriocin. *S. aureus* ATCC 25923 was less sensitive to bacitracin and amikacin than bacteriocin. *Escherichia coli* ATCC 35218 was resistant to both bacteriocin and antibiotics except gemifloxacin. Furthermore, the efficacy of bacteriocin against *E. faecalis* ATCC 29212 was lower than gemifloxacin but higher than gentamicin, amikacin, bacitracin, ciprofloxacin and ampicillin. It is obvious from the Figure 6 that



**Figure 5:** Molecular weight determination of partially purified bacteriocin compared with molecular weight marker (MWM) by SDS-PAGE. Arrow indicates the *in situ* activity of partially purified bacteriocin 28D1.

*Enterococcus* spp. (hospital waste isolate) was sensitive to bacteriocin but resistant to all the antibiotics. On the other hand, *Bacillus* spp. (hospital waste isolate) was almost equally sensitive to the bacteriocin, gemifloxacin, and gentamicin but the bacteriocin was required at a very low dose (3.2 µg) to get the desired result.

#### Comparison of antibacterial activity of bacteriocin with antibiotics based on MIC

The MIC of antibiotics were interpreted and finalised as sensitive, moderately sensitive and resistant as per CLSI guidelines, 2018. As seen in Table 5, *E. coli* ATCC 25922 was most sensitive to ciprofloxacin (MIC  $0.01 \pm 0.00$  µg/mL), gemifloxacin (MIC  $0.01 \pm 00$  µg/mL), bacteriocin (MIC  $0.16 \pm 0.07$  µg/mL) and moderately sensitive to other antibiotics. Moreover, *E. coli* ATCC 35218 was highly sensitive to gemifloxacin (MIC  $0.01 \pm 00$  µg/mL) but resistant to the other antibiotics including the bacteriocin. Similarly, *S. aureus* ATCC 25923 was highly sensitive to gemifloxacin (MIC  $0.01 \pm 00$  µg/mL), bacteriocin (MIC  $0.06 \pm 0.00$  µg/mL), ciprofloxacin (MIC  $0.25 \pm 0.00$  µg/mL), ampicillin (MIC  $0.33 \pm 0.14$  µg/mL) and gentamicin (MIC  $0.33 \pm 0.14$  µg/mL). The MIC of bacteriocin was high i.e.,  $0.06 \pm 0.00$  µg/mL for *S. aureus* ATCC 25923 as compared to MIC of gemifloxacin ( $0.01 \pm 0.00$  µg/mL) but lower than the other antibiotics. Also, *P. aeruginosa* ATCC 27853 was highly sensitive to ciprofloxacin (MIC  $0.42 \pm 0.14$  µg/mL) in this study. The MIC of bacteriocin ( $3.33 \pm 1.40$  µg/mL) for *P. aeruginosa* ATCC 27853, which was higher than the MIC of ciprofloxacin ( $0.42 \pm 0.14$  µg/mL),

gemifloxacin ( $0.50 \pm 0.00$  µg/mL) and bacitracin ( $0.83 \pm 0.28$  µg/mL), almost equal to amikacin ( $4.00 \pm 0.00$  µg/mL) and lower than all other antibiotics. Furthermore, *E. faecalis* ATCC 29212 showed highest sensitivity to gemifloxacin (MIC  $0.05 \pm 0.02$  µg/mL) followed by the bacteriocin (MIC  $0.12 \pm 0.00$  µg/mL) and ciprofloxacin (MIC  $0.50 \pm 0.00$  µg/mL). The sensitivity to the rest of the antibiotics was markedly low as made out by the high MIC values.

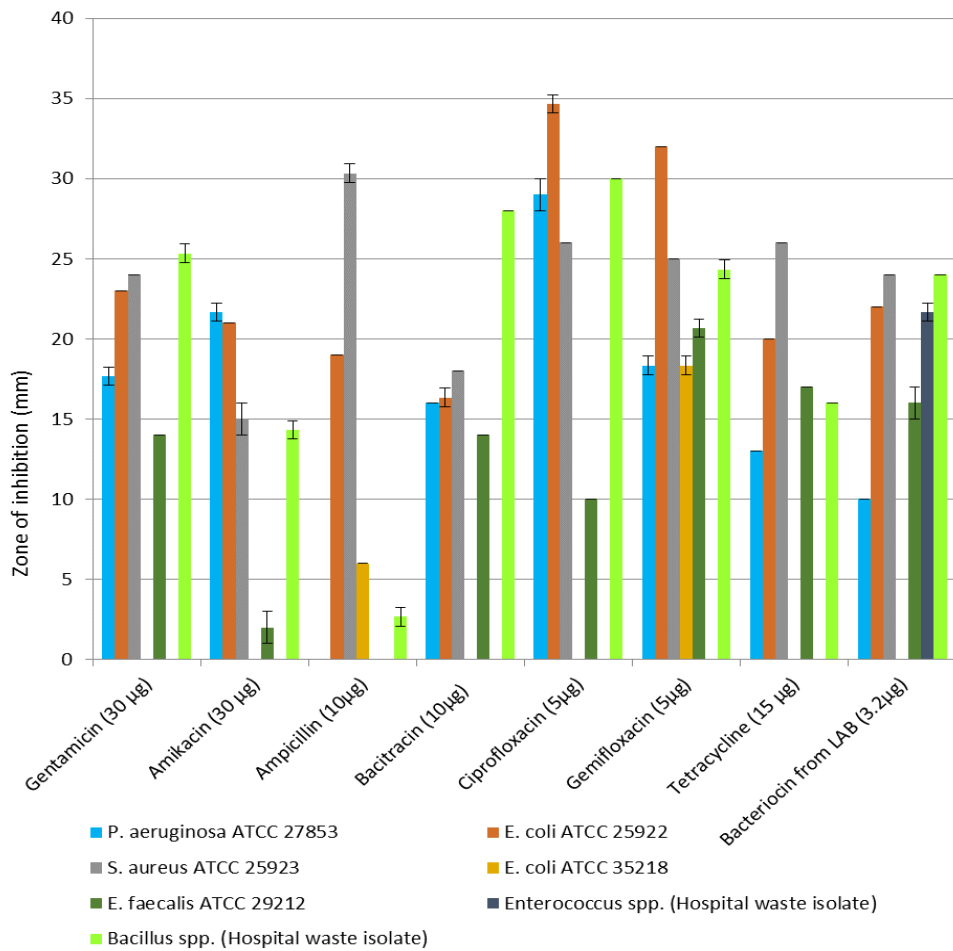
Surprisingly, *Enterococcus* spp. (hospital waste isolate) showed the highest sensitivity to bacteriocin (MIC  $0.06 \pm 0.00$  µg/mL) but was resistant to all others antibiotics tested in this study (Figure 7). On the other hand, *Bacillus* spp. (hospital waste isolate) showed highest sensitivity to ciprofloxacin (MIC  $0.02 \pm 0.01$  µg/mL) followed by bacteriocin (MIC  $0.03 \pm 0.00$  µg/mL), bacitracin (MIC  $0.04 \pm 0.02$  µg/mL), gentamicin (MIC  $0.42 \pm 0.14$  µg/mL), gemifloxacin (MIC  $0.50 \pm 0.00$  µg/mL) and tetracycline (MIC  $1.00 \pm 0.00$  µg/mL) respectively. It was resistant to amikacin and ampicillin.

#### DISCUSSION

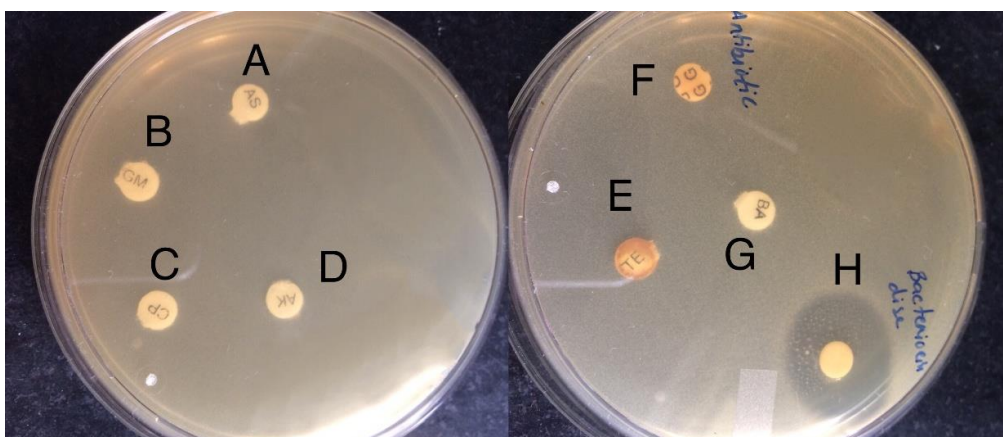
LAB is known to have strong antimicrobial activity against various pathogens which have been identified as causative agents for the deterioration of food as well as various human diseases. In the present study, out of 38 LAB isolates from curd, 28D1 showed the highest antimicrobial activity against indicator strain *E. faecalis* NCDC 114. Hence, *E. faecalis* NCDC 114 was chosen as the best indicator strain for this study. This was based on the results of sensitivity tests and diameter of zone of inhibition which indicated that *E. faecalis* was sensitive to almost all the LAB isolates during this study. The study agreed with the report of Pandey (2013) who also found *E. faecalis* NCDC 114, the best indicator strain.

The crude bacteriocin was extracted from 28D1 and purified partially by ASP. After partial purification, the total activity unit of the bacteriocin was estimated to be 3,072,000 AU/mL and the overall yield was 120%. The method, ASP is versatile for concentrating antimicrobial proteins from the CFCS (Alam, 2010). The purification of bacteriocin involves the separation of the bacteriocin from the unwanted proteins present in the CFCS. The yield and purity of bacteriocin obtained by this method is influenced by the amount of unwanted protein present in the sample that is being purified which in turn leads to a difference in the end purity and yield of purified bacteriocin. The present results completely agreed with Singh *et al.* (2013) who partially purified bacteriocin from *Lactobacillus fermentum* SBS001 using above mentioned method. As per work done by different scientists, partially purified bacteriocin was obtained at 80% ASP (Ivanova *et al.*, 2000; Cherif *et al.*, 2008; Feliatra *et al.*, 2018). However, scientists have claimed that partially purified bacteriocin was obtained at 40% ASP (Muriana and Klaenhammer, 1991), 60% ASP (Bello *et al.*, 2018) and 70% ASP (Sure *et al.*, 2016).

Besides, the crude bacteriocin was found to be stable over a wide range of temperatures (60-100°C) and pH (4-



**Figure 6:** Comparing the efficacy of antibiotics with bacteriocin on indicator bacterial strains by disc diffusion method. The data represents mean value of triplicate experiments and the error bars represent the standard deviation.



**Figure 7:** Antimicrobial activity of partially purified bacteriocin and antibiotics against *Enterococcus* spp. (hospital waste isolate) by disc diffusion assay. A, Ampicillin (10 µg); B, Gentamicin (30 µg); C, Ciprofloxacin (5 µg); D, Amikacin (30 µg); E, Tetracycline (15 µg); F, Gemifloxacin (5 µg); G, Bacitracin (10 U); H, Bacteriocin (3.2 µg).



**Table 5:** Determination of MIC ( $\mu\text{g/mL}$ ) of antibiotics and bacteriocin on indicator bacterial strains.

Antibiotics	MIC ( $\mu\text{g/mL}$ )*						
	ATCC strains				Hospital waste isolates		
	<i>E. coli</i> ATCC 25922	<i>E. coli</i> ATCC 35218	<i>S. aureus</i> ATCC 25923	<i>P.</i> <i>aeruginosa</i> ATCC 27853	<i>E. faecalis</i> ATCC 29212	<i>Enterococcus</i> spp.	<i>Bacillus</i> spp.
Gentamicin	0.50 $\pm$ 0.00	>64	0.33 $\pm$ 0.14	2.00 $\pm$ 0.00	6.66 $\pm$ 2.30	>64	0.42 $\pm$ 0.14
Amikacin	1.33 $\pm$ 0.57	>128	2.66 $\pm$ 1.15	4.00 $\pm$ 0.00	85.33 $\pm$ 36.95	64.00 $\pm$ 0.00	8.00 $\pm$ 0.00
Ampicillin	4.00 $\pm$ 0.00	16.00 $\pm$ 0.00	0.33 $\pm$ 0.14	>256	>256	>256	170.66 $\pm$ 70.90
Bacitracin	8.00 $\pm$ 0.00	>256	2.66 $\pm$ 1.10	0.83 $\pm$ 0.28	4.00 $\pm$ 0.00	>256	0.04 $\pm$ 0.02
Ciprofloxacin	0.01 $\pm$ 0.00	>32	0.25 $\pm$ 0.00	0.42 $\pm$ 0.14	0.50 $\pm$ 0.00	>32	0.02 $\pm$ 0.01
Gemifloxacin	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.50 $\pm$ 0.00	0.05 $\pm$ 0.02	2.00 $\pm$ 0.00	0.50 $\pm$ 0.00
Tetracycline	2.00 $\pm$ 0.00	>256	42.00 $\pm$ 0.14	16.00 $\pm$ 0.00	13.33 $\pm$ 4.61	>256	1.00 $\pm$ 0.00
Bacteriocin from LAB	0.16 $\pm$ 0.07	>16	0.06 $\pm$ 0.00	3.33 $\pm$ 1.40	0.12 $\pm$ 0.00	0.06 $\pm$ 0.00	0.03 $\pm$ 0.00

\* Mean of MIC ( $\mu\text{g/mL}$ ) value of triplet sets of experiments.

9). The approximate molecular weight of the bacteriocin was determined as 8.5 kDa. It was previously reported that the proteinaceous composition of bacteriocin L23 from *L. fermentum* was confirmed by the inhibition of bacteriocin when it was subjected to the action of proteolytic enzymes. Catalase and lipase did not affect the bacteriocin. The bacteriocin activity was adversely affected when it was subjected to extremes of temperature and pH. The bacteriocin activity was maximum at pH of 5 and stable between a pH of 3-7. There was deterioration in activity above and below this pH (Yan and Lee, 1997; Pascual *et al.*, 2008). Another characterization study carried out on the bacteriocin from *Lactobacillus* spp. gave evidence of temperature and pH (2-6) stability of bacteriocin (Mojgani *et al.*, 2009). Recently, researchers from Howard University had shown that probiotic isolated from yoghurt had strong antimicrobial activity. Among the multiple *Lactobacillus* isolated, they studied *Lactobacillus parafarraginis* which had an exceptional molecular weight of 75 kDa (Allen-McFarlane *et al.*, 2019). This is significant because most of the bacteriocin possess a very low molecular weight of less than 10 kDa (Dicks *et al.*, 2018).

Our work compares the antibacterial action of partially purified bacteriocin with that of antibiotics against some indicator strains. According to the present study, bacteriocin from LAB showed good bactericidal action against most of the indicator strains except *E. coli* ATCC 35218 (Table 5). The present result demonstrates that gemifloxacin was the best-suited antibiotic which showed strong inhibition to most of the indicator strains except *Enterococcus* spp. (hospital waste isolate). Hardy *et al.* (2000) also reported that gemifloxacin was the best studied antibiotic among 9 different antibacterial agents

against different indicator strains. It was found that *E. coli* ATCC 35218 and *Enterococcus* spp. (hospital waste isolate) were resistant to most of the antibiotics. Antibiotic resistance was also found in *E. coli* ATCC 35218 which was reported by Karatuna (2012). *Enterococcus* spp. (hospital waste isolate) was found to be multi-drug resistant but sensitive to bacteriocin from LAB (Table 5 and Figure 7). The isolated and partially purified bacteriocin from LAB has a broad spectrum of antimicrobial activity at a much lower concentration than most antibiotics and it is very effective (MIC 0.06  $\pm$  0.00  $\mu\text{g/mL}$ ) against hospital-acquired multidrug-resistant *Enterococcus* spp.

## CONCLUSION

In conclusion, our study demonstrates that bacteriocin from LAB would be a successful alternative to antibiotics against MDR infections particularly enterococcal infections. The identified bacteriocin was found to be stable over a wide range of pH and temperature. The findings of this study are particularly significant in establishing the therapeutic potential of bacteriocins in tackling human infections in the near future.

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