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Combat of Bacteriocin Producing *Lactiplantibacillus pentosus* LMEM1001 isolated from Neera with Bactericidal activity against MRSA and VRSA Strains

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ABSTRACT: Antimicrobial resistance accelerates due to overuse and indiscriminate use of antibiotics by all living creature. Indiscriminate use of antibiotics and other chemical compounds leads to several side effects and results in increased antibiotic resistance day by day. The emergence of antibiotic-resistant bacterial strains, including Extended-Spectrum Beta-Lactamase (ESBL), Methicillin-Resistant Staphylococcus aureus (MRSA), and Vancomycin-Resistant Staphylococcus aureus (VRSA), has become a significant global health concern. To address this issue, alternative antimicrobial agents are being explored, and bacteriocins produced by lactic acid bacteria are gaining attention due to their potential as natural alternatives to conventional antibiotics. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria, harmless to human and more effective for gastroinstestinal tract infections. Study investigates the bactericidal activity of bacteriocins from by *Lactiplantibacillus pentosus* LMEM1001 (VRN-32) isolated from Neera a traditional probiotic drink. Bacteriocin production was confirmed by well diffusion assays. Bacterioicin from VRN-32 was purified by ammonium sulphate precipitation, and RP-HPLC. Partially Purified bacteriocin was stable against heat and extreme pH. Inhibitory effects of bacteriocin found to be stable over a broad temperature 80°C and pH 8.0 Bacterioc in was stable with Lysozyme and resistant to proteinase-K. Stability of bacterioic in was maximum with surfactants such as Tween-80 and Triton X-100, where as SDS and β-mercaptoethanol found to decrease its stability. Bacteriocin from VRN-32 showed promising antimicrobial activity against Methicillin Resistant *Staphylococuss aureus* (ATCC- 33591) and Vaccomycin Resistant *Staphylococuss aureus* VRSA stains isolated from hospital acquired infections. Lantibiotic isolated from probiotic drink neeera was partial purified and showed promising activity against emerging Super Bugs and the probiotic found to be an excellent alternative to hazardous antibiotics.

Keywords: Antimicrobial resistance, Neera, MRSA, VRSA.

INTRODUCTION

World is facing serious problems due to pathogenesis and microbial adhesion resulting in antimicrobial resistance. The longevity of antibiotics in the medical sector is a threat due to rapid emergence and spread of bacterial resistance (Elaine *et al*., 2020). According to World Health Organization (WHO), more than 7lakh persons end their lives every year drug-resistant which will increase by 2050 to 10million when no action taken (WHO, 2020). Overuse and misuse of antimicrobial drugs leads to AMR to promote growth and prevent disease in livestock, which contributes to the development of drug-resistant bacteria that can spread to humans (Center for Disease Control and Prevention, CDCP 2021). Anti Microbial Resistance (AMR) is a global public health threat that affects people of all ages and in all parts of world that could nullify the progress made in modern medicine. Though antibiotics are playing a pivotal role but their excess use in intensive animal production has several side effects with antibiotic resistance. Therefore, scientists are looking for an alternative to antibiotics which have less side effects and more effective. Bacteriocin are natural antimicrobial peptides produced by bacteria, making them a sustainable and eco-friendly, also play a significant role and have potential applications.

Bacteriocins being heterogeneous group some are extensively post-translationally modified ribosomally synthesized antimicrobial peptides produced by bacteria having probiotic trait and as natural compounds able to influence the safety and quality of food. These probiotic bacteria are advantageous and due to the fact that they harbor the gut of humans hence are advantageous (Kelsic *et al.*, 2015). Thus, represent potential alternative to traditional antibiotics with high potency, low toxicity can be utilized in bioengineering without side effects. Bacteriocins have been classified into four major classes, based on their molecular structure and mode of action: Class I (lantibiotics), Class II (non-

lantibiotics), Class III (large heat-labile proteins), and Class IV (complex bacteriocins). (Klaenhammer *et al.,* 2009).

Infections with MRSA, and VRSA are associated with increased morbidity, mortality, and prolonged hospital stays.

MRSA infections can vary depending on the site of infection, the health status of the individual, and the presence of other risk factors. MRSA is a strain of *Staphylococcus aureus* bacteria that has developed resistance to multiple antibiotics, including methicillin and other beta-lactam antibiotics (CDC, 2019). Emergence of VRSA is concerning because it limits treatment options and can lead to increased mortality rates. Severity of VRSA infections is similar to that of MRSA, ranging from mild to severe, with the potential for life-threatening complications VRSA is a strain of Staphylococcus aureus that has acquired resistance to the antibiotic vancomycin, which is often considered the "last-resort" antibiotic for treating severe *Staphylococcal* infections (CDC, 2020).

Scientists have been studying bacteriocins as potential alternatives to antibiotics in the fight against multidrug-resistant bacteria. Some bacteriocins have shown promising activity against MRSA, VRSA strains. It's important to note that while bacteriocins hold potential, they are silver bullet solution. Combating antibiotic resistance requires a multifaceted approach, including the development of new antibiotics, improvement of infection control practices, and the prudent use of existing antibiotics to minimize resistance selection pressure. Bacteriocins are considered as natural alternatives to antibiotics for the treatment of bacterial infections. The stability of bacteriocins is crucial to ensure their effectiveness in vivo. If bacteriocins are unstable and rapidly degrade or lose activity under physiological conditions, their therapeutic efficacy may be compromised.

The aim of this study is to investigate the bacteriocin produced by *Lactiplantibacillus pentosus* LMEM1001 (VRN-32) isolated from Neera, is a traditional probiotic drink and its potential effectiveness against Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant *Staphylococcus aureus (VRSA),* strains. The isolated bacteriocin was purified high-performance liquid chromatography (HPLC). Characterization of purified bacterioicin was to determine its stability, optimum pH, temperature range, and sensitivity to proteolytic enzymes. The findings from this study could contribute to the development of alternative antimicrobial strategies to combat multidrug-resistant bacteria, particularly MRSA and VRSA, strains. Understanding the potential of *Lactiplantibacillus pentosus* LMEM1001 (VRN-32) derived bacteriocins may provide valuable insights for future therapeutic applications in the fight against antibiotic-resistant pathogens

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria (LAB). Isolation of LAB was done from Neera - a probiotic drink (Poornachandra *et al.,* 2015). Fresh neera was collected (early morning before 6am) from Karnataka border Hyderabad Telangana state. One ml of each sample was inoculated on De Man Rogosasharpe agar (MRS) and plates incubated at 37°C for 24h. Morphologically discrete colonies were selected, subcultured on MRS agar. Only catalase negative, gram positive colonies were LAB separated and used further.

Preliminary Screening of Bacterioicn Producers. Antimicrobial activity of isolate (VRN-32) was done by disc diffusion method. Isolate LAB was inoculated in MRS broth and incubated at 37°C incubated at 37°C for 48h (Deshmukh *et al.,* 2013). Fermented broth was centrifuged at 12,000rpm for 15min at 4°C passed through 0.22µm filter. Antimicrobial activity of cell free supernant against *E.coli*(ATCC8739),*P. aeruginosa* (ATCC9027), *Klbesiella pneumonia, Proteus vulgari, S. epidermis*(ATCC12228) *S. aureus*(6538), *Streptoccocus faecalis*(8043), *Bacillus subtills* (ATCC-6633), *Candida glabrata, C. haemulone, C. albicans* (ATCC-10231)activity was checked by disc diffusion method which was made on Muller Hinton agar previously seeded with 18h old culture of pathogens which was grown in nutrient broth medium and incubated at 37°C was diluted equivalent to that of 0.5 McFarland standard (Eliane *et al.,* 2016).

Activity of Bacterioicin Produced from VRN-32. Isolate VRN-32 was inoculated in MRS broth and was incubated at 37°C at 120 rpm for 48h. Fermented broth was centrifuged at 12,000rpm for15min, supernant was collected 7.0 pH was set. Ammonium sulphate precipitation was done at saturation rate of 70% and stored at 4°C. After precipitation, broth was collected and centrifuged at 15,000rpm for 15min and stored in 0.2M sodium phosphate buffer (pH-6.9) which is crude bacterioicin (Navarro *et al.,* 2000). Antimicrobial activity against pathogens with crude precipitate was done by disc diffusion method which confirms the presence of antimicrobial substance (Deshmukh *et al.,* 2013).

Extraction of Bacteriocin from VRN-32. Crude bacteriocin was extracted by Chloroform: Methanol (2:1V/V). Produced precipitate at solvent aqueous interphase was collected aseptically, excess solvent was evaporated and precipitate was kept in buffer, later used for antimicrobial study (Burianek *et al.,* 2000).

Molecular Characterization of bacteriocin producing VRN-32. Preliminary identification of LAB isolates was based on phenotypic and biochemical characteristics which includes Gram's stain reaction, cell morphology, catalase test, oxidase standard morphological and biochemical characteristics described in Bergey's manual of systematic bacteriology. Molecular identification of LAB was determined by 16SrDNA sequencing. Genomic DNA was isolated from the sample. The ~1.5 kbp, 16s-rDNA fragment was amplified using high–fidelity PCR polymerase. The PCR product was sequenced bi-directionally. The obtained homologous sequences, were searched in using the Basic Local Alignment Search Tool (BLAST) and sequence was submitted to GenBank sequence database and accession number was obtained.

Purification of Bacteriocin from VRN-32. Purity of bacteriocin was determined by HPLC equipped with a chromatographic column of SB-C18.Bacteriocin was loaded into HPLC column. Solvent A (Water: Acteonitrile=1:99 Himedia) and solvent B (Acteonitrile 100%) were used. Gradient elution was programmed as follows: initial solution ratio was A: B=1:10 for 10min, 10-30min for gradient elution, the final solution ratio was A: B=0:1, solution flow rate was 0.8ml/min, column temperature was 25°C. Sample was monitored at 280nm. Active fraction was collected and antimicrobial activity was done by disc diffusion method (Zhang *et al.*, 2018).

Stability of Enzyme on Bacteriocin Activity from VRN-32. Partially purified bacteriocin was incubated for 2h at 37°C with 1mg/ml of enzymes such as Trypsin, Proteinase-K, α-Amylase, Pepsin, Cellulase, and Lysozymes. Antimicrobial activity of bacteriocin was checked (Todorov *et al.*, 2012).

Stability of Bacteriocin from VRN-32 at Different pH's and Temperatures. Partially purified bacteriocin was subjected to different pH's and temperatures to check its stability. The pH of partially purified bacteriocin was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 using HCl and NaOH. Bacteriocin was heated at 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 121°C for a time period of 30min. Stability and its activity after heat treatment and pH change were checked and tabulated (Ogunbanwo *et al.*, 2003). Antimicrobial activity of sample was checked by agar well diffusion assay.

Stability of Detergents and Surfactants on Bacteriocin Activity from VRN-32. Detergents and surfactants such as EDTA, SDS, Tween-80, Tween-20, TritonX-100, NaCl, β-mercaptoethanol at concentrations of 1% w/v were added individually into partially purified bacteriocin. All tubes were incubated at 37°C for 2h. Antimicrobial activity of bacteriocin VRN-32 was checked by agar well diffusion assay (Todorov *et al.*, 2012).

Antimicrobial Activity of Bacteriocin against Vancomycin-Resistant *Staphylococcus aureus* (VRSA) and Methicillin Resistant *Staphylococus aureus* (MRSA).

Vaccomycin-resistant *S.aureus* was isolated from hospital acquired infection, and was resistant to Vancomycin antibiotic and Methicillin Resistant (MRSA)(ATCC-33591) was collected from culture collection center was resistant to Methicillin. Antimicrobial activity of bacteriocin was checked against both MRSA and VRSA by disc diffusion method. Both the *S.aureus* strains were grown in BHI broth and swabbed on Muller Hinton agar plate and wells of 6mm were made using sterile cork borer. Partially purified Bacteriocin(100µl) was inoculated in each well, and plates were incubated overnight at 37°C for 18-24h for zone of inhibition (Naveed *et al.*, 2013).

RESULTS AND DISCUSSION

Isolation and Characterization of LAB from Probiotic Drink Neera. Among 50 isolates 40 showed zone of inhibition of which three isolates VRN-32, VRN-17, VRN-25 showed good zone of inhibition. Among them VRN-32 showing maximum zone of inhibition was further studied. Isolate VRN-32 was rod shaped, gram positive, negative for oxidase and catalase test (Table 1). Isolate VRN-32 was genomically identified as *Lactiplantibacillus pentosus strain* LMEM1001(Accession no: ON692896). The sequences obtained was deposited in Genbank using NCBI and accession number was obtained (Table 2) (Fig.1 a&b). Objective of the study was to isolate and identify bacterioicin producing *L. pentonsus* LMEM1001 (VRN-32) isolated from traditional probiotic drink neera and study their resistant pattern. Several *L. pentosus* stains have been isolated from many different sources, *L.pentosus* RL2e (Tek *et al.*, 2015) *L. pentosus* (Okkers *et al.*, 1999), *L. pentosus* COORG8, *L. pentosus* COORG3 (Poornachandra *et al.*, 2015) and *Pediococcus pentosaceous* (Ramos *et al.*, 2016), *L .pentosus* ST712BZ (Svetoslav *et al.*, 2007), *L. pentosus* (Jiong *et al.*, 2017). Similarly, to the previous studies the present study *L. pentosus* LMEM1001 was isolated from Neera which have high antimicrobial activity against several pathogens.

Table 1: Biochemical Characteristics of the Isolate (VRN-32).

Test performed	Observation
Morphology	Rods
Gram Staining	Positive
Motility	Negative
Voges Proskauer	Positive
Catalase Test	Negative
Citrate Utilization Assay	Negative
Gas Production	Positive
Oxidase	Negative

Table 2: Genomic Identification of bacteria VRN-32.

BLAST results	Gen bank accession number
Lactiplantibacillus pentosus strain LMEM1001	ON692896

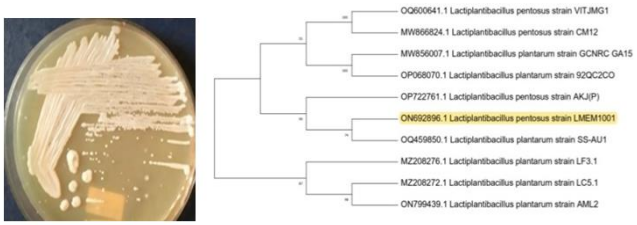

Fig. 1(a) Colony morphology of LAB isolate on MRS Agar (b) Phylogenetic tree of the LAB strains obtained by molecular phylogenetic analysis.

Antimicrobial Activity of Bacteriocins from *L. pentosus* LMEM1001 VRN-32. Antimicrobial activity of *L. pentosus* LMEM1001 VRN-32 was tested against pathogenic bacteria and fungi. Antimicrobial susceptibility of crude bacterioicin showed inhibitory activity against *E. coli* (ATCC8739), *P. aeruginosa* (ATCC9027*), K. pneumonia, P. vulgaris, S. epiderm dis* (ATCC12228), *S. aureus (6538), P. plecoglossicida, B. subtils* (ATCC6633), *Streptococcus faecalis (8043),C. albicans* (ATCC-10231), *C. glabrata, C. haemulone* zone of 16-18mm (Table 3 Fig. 2). Several studies with bacteriocins from *L. pentosus* RL2e were tested against *E. coli, S. auerus, P. aerouginosa, L. monocytogenes, Yersinia pestis, Shigella,* they reported minimum zone of inhibition 2-4mm with *B. cerus, E. coli, S. auerus, Yersinia pestis* and *S. dynesterial*, whereas with *P. aerouginosa, L. monocytogenes* it showed nil inhibition (Tek *et al.,* 2015). Similarly *E. faecalis KT11* showed zone of inhibition (14 to 20mm) against gram negative bacteria (Hilal *et al.,* 2016). *L. pentosus* COORG 3 & 8 showed zone of inhibition which ranged from 4-17mm and the strains COORG5, COORG7, and COORG8 showed zone of inhibition 4-17mm and the strains COORG5, COOR7 and COOR 8 showed zone of 15, 17, 16mm against L-*monocytogens and E. fecailis* (Poornachandra *et al.,* 2015). Okkers *et al.* (1999) showed a zone of 10-15mm against *C. albicans.* Our isolate *L. pentosus* LMEM1001 showed a good antimicrobial activity of 12-18mm against almost similar organism both bacteria and fungi. Comparing the antimicrobial studies with other workers our bacteriocin from *L. pentosus* LMEM1001 appears to be more effective against deadly pathogens due to the fact that the zone of inhibition is about 10 times more.

Table 3: Bacteriocin from *L. pentosus* LMEM1001 showing Zone of inhibition against pathogens.

Test organism	Zone of inhibition(mm)
E. coli (ATCC-8739)	16mm
Ps. Aeruginosa (ATCC-9027)	14mm
K. pneumonia	15mm
P. vulgaris	15mm
S. aureus (6538)	15mm
S. epidermidis (ATCC-12228)	15mm
S. faecalis (8043)	13mm
P. plecoglossicida	16mm
B. subtilius	18mm
L. monocytogenes (ATCC 19112)	12mm
C. haemulonei	14mm
C. glabrata,	17mm
C. albicans (ATCC-10231)	12mm

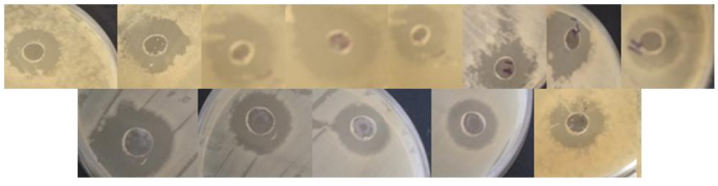

Fig. 2. Zone of inhibition by different pathogens bacteriocin from *L. pentosus* LMEM1001.

Partial Purification of Bacteriocin by HPLC and its Activity. Partial purification of bacterioicin produced by VRN-32 was performed by Ammonium sulphate precipitation and RP-HPLC. During RP-HPLC single peak was collected at 2.5min showed maximum protein concentration (Fig. 3) and single peak revealed the presence of inhibitory compound and antimicrobial activity of the same fraction was performed with test organisms and showed zone of inhibition between 27-13mm (Table 4). Our results correlate with Dai *et al.*, where he reported zone of 22-25mm, against test organisms, isolated from *L. pentosus* ZFM94 (Dai *et al.*, 2021).

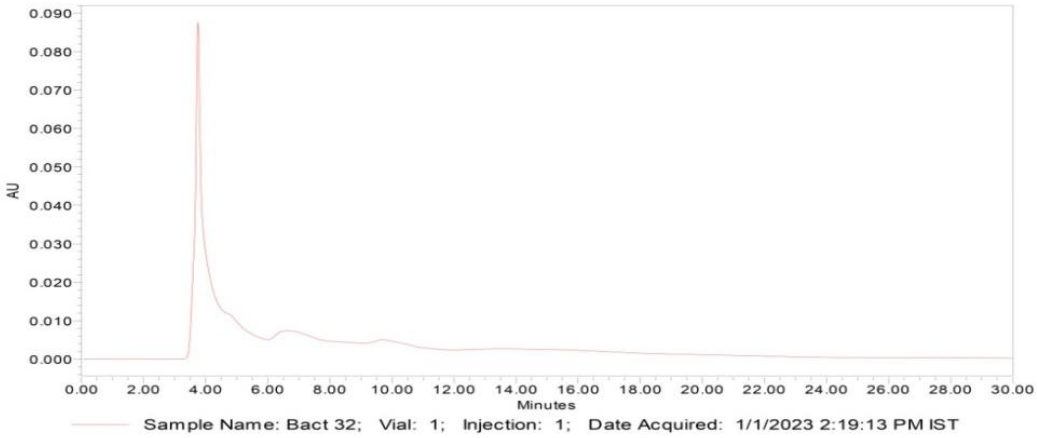

Fig. 3. HPLC chromatogram of purified bacteriocin.

Table 4 : Bacteriocin Produced by *L. pentosus* strain LMEM1001 showing Zone of Inhibition.

Test organism	Zone of inhibition (mm)
E. coli (ATCC-8739)	22mm
P. aeruginosa (ATCC-9027)	22mm
K. pneumonia	27mm
P. vulgaris	31mm
S. aureus (6538)	15mm
S. epidermidis (ATCC-12228)	15mm
S. faecalis (8043)	13mm
P. plecoglossicida	18mm
B. subtilius	18mm
L. monocytogenes (ATCC 19112)	16mm
C. haemulonei.	16mm
C. glabrata,	19mm
C. albicans (ATCC-10231)	16mm

Effects of Temperature, pH, Enzymes, Surfactant and Detergents on Bacteriocin. Bacteriocin was treated with proteolytic enzymes, Proteinase-K, Lysozymes, Pepsin, Trypsin, α-Amylase, Cellulase and bacteriocin activity was determined against *S. aureus* (6538), *E. coli (ATCC-8739), K. pneumoniae, S. epidermidis* (ATCC-12228). In our study bacterioicin was resistant to proteinase-K, while Lysozyme stability was more compared to Trypsin and Pepsin, whereas α-Amylase and Cellulase showed the same stability there was no change in activity (Table 5). The antimicrobial activity from bacterioicin isolated from traditionally fermented products has no effect when treated with proteoytic enzyme except pepsin (Dai *et al.,* 2021). Study showed reduction in activity when treated with other proteases (typsin and pepsin) and activity was enchanced by Lysozymes whereas Amylase and Cellulase did not affect the stability of bacteriocin produced by *L. pentosus* ST712BZ (Todorov *et al.* (2007). However, it is clear that enzymes can play an important role in determining activity and stability of bacteriocins produced from bacterium *L. pentosus* in general.

In our study bacteriocin produced by *L. pentosus* LMEM1001 remained highly stable at the temperatures ranging from 20°-80°C and stability was decreased from 90-121°C (Fig. 4). Biswas *et al.* (2017) reported that bacteriocin was stable at 60°-80°C for 60min and stability was lost at 100 and 121vC. In our study, bacterioicn produced by *L. pentosus* LMEM1001 was stable upto 60 min at pH 2.0–10.0, but its activity decreased significantly at pH 10.0 and was completely lost at pH 12.0 (Fig. 5). Similar results were reported with Biswas et.al., where bacteriocin was stable at pH 2.0-10.0 (Biswas *et al.*, 2017). Different LAB strains antimicrobial activity ranged from pH 4.0 to 8.0 (Heredia-Castro *et al.*, 2021).

Different concentration of surfactants and detergents were used to study the bacteriocin activity from *L. pentosus* LMEM1001. Bacteriocin was supplemented with SDS, Tween-80, Tween-20, TritonX-100, EDTA, β-mercaptoethanol and NaCl and found that surfactants, such as Triton X-100, and Tween-80, improved stability of bacteriocin while, SDS and β-mercaptoethanol, decreased its stability. EDTA and Tween-20, had no effect on its activity while, NaCl increased the activity (Fig. 6). where stability of bacteriocin with Tween 80 and Triton X-100 (Radha *et.al.,* 2015) while others, SDS and CTAB, decreased the stability (Biswas *et al.,* 2017). Bacterocin activity was inhibited after treatment with EDTA and urea whereas SDS, Tween-80, Tween-20, and Triton-X stimulated bacteriocin activity (Aslam *et al.,* 2012).

Table 5: Effects of enzyme stability on bacterioicin production.

Enzymes	Zone of inhibition (+++, ++, +)			
	E. coli (ATCC-8739)	*K. pneumonia*	*S. aureus (ATCC-6538)*	*S. epidermidis* (ATCC-12228)
Proteinase-K,	-	-	-	-
Trypsin	++	+++	+++	++
pepsin,	++	+++	+++	+
α-Amylase,	++	++	+	+
Lysozymes	+++	+++	+++	_
cellulase	++	++	++	-

(+++ =zone of inhibition 25mm, ++=15mm, --= no zone of inhibition)

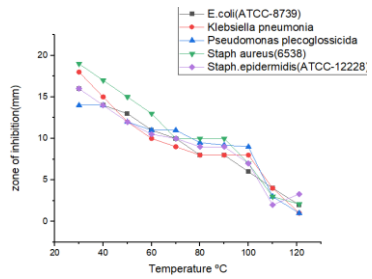

Fig. 4. Effects of Temperature.

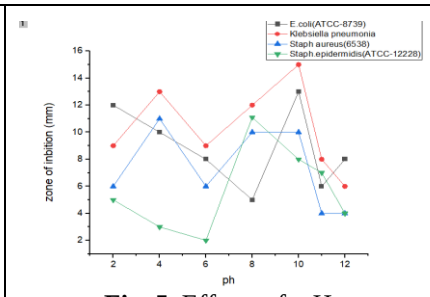

Fig. 5. Effects of pH.

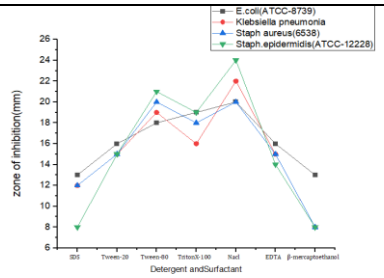

Fig. 6. Effects of Surfactants and Detergent.

Antimicrobial Activity of Bacteriocin against MRSA and VRSA. Bacteriocin producing *L pentosus* LMEM1001 showed antimicrobial activity against of MRSA and VRSA. Sensitive strains of MRSA showed 24mm zone and VRSA showed 26mm zone (Fig.7a, b, c, d). Nisin from *L. lactis* had shown a zone of inhibition of 18 mm against MRSA and 16 mm against VRSA (Yehia *et al.*, 2021). Nisin had strong antimicrobial activity, with zone size ranging from16mm against MRSA (Brumfitt *et al*., 2002).

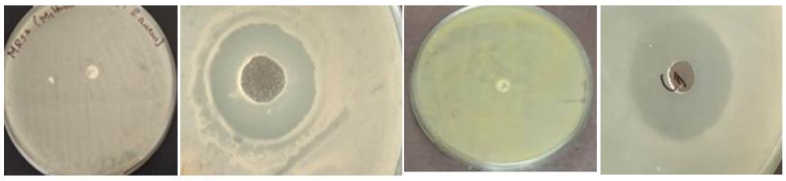

Fig. 7. (a, b) Confirmation of MRSA and Zone of inhibition bacteriocin producing *L. pentosus* LMEM1001 c, d) Confirmation of VRSA and Zone of inhibition by bacteriocin producing *L. pentosus* LMEM1001

CONCLUSIONS

Antimicrobial peptides called bacteriocins produced by bacteria and have the ability to either kill or stop the growth of other bacteria. Bacteriocins hold great promise as a new class of antibiotics and food preservatives, but more research is needed to fully understand their potential and to develop effective methods for their production and application. With the increasing threat of antibiotic resistance a new viable alternative is necessary. Our bacteriocins isolated from *L. pentosus* LMEM1001 have broad inhibitory action against gram positive, gram negative and fungal pathogens and it is stable at high temperature and low pH, which can be used in biopreservation and medical fields as bacteriocin are effective against MRSA,VRSA strains. Hence, bacteriocin produced by *L. pentosus* LMEM1001 can represent a promising alternative that could help combat the spread of infectious diseases and improve food safety and control diseases caused due to pathogens with nil side effects.

FUTURE SCOPE

The discovery of *Lactiplantibacillus pentosus* LMEM1001, a bacteriocin-producing strain with bactericidal activity against MRSA (Methicillin-Resistant Staphylococcus aureus), and VRSA (Vancomycin-Resistant *Staphylococcus aureus*) strains, holds significant promise for combatting antibiotic-resistant bacteria. *Lactiplantibacillus pentosus* LMEM1001's bacteriocin could serve as a valuable template for the development of new antibiotics or antimicrobial agents targeting ESBL, MRSA, and VRSA strains. Further research and optimization of this bacteriocin could lead to the creation of more potent and specific therapeutics.

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Conflict of Interest. None.

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