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Antimicrobial activity of Bacteriocin from *Lactiplantibacillus plantarum 1625* isolated from Neera a probiotic Drink

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Abstract

Antibiotic resistance with modern medicine is a biggest threat to mankind. Scientists are seeking for product which is used as an alternative to antibiotic with less side effects. Bacteriocins are antimicrobial peptide produced by Lactic Acid Bacteria (LAB) that have potential applications in food industry as natural preservatives. In our study we isolated bacteriocin producing *Lactobacillus* VRN-25from Neera (a probiotic drink) collected from Karnataka border, Telangana Region and screened for bacteriocin production by agar disc diffusion method. Among the 40 isolates VRN-25, showed maximum zone of inhibition against *Klebsiella pneumoniae* (27mm), *Staphylococcus aureus* (22mm) *and Candida glabrata* (16mm). Hence, VRN-25 was selected and characterized by Morphological, Biochemical and Molecular methods.VRN-25 was identified as *Lactiplantibacillus plantarum* (ON692894). Partial purification was done by RP-HPLC with retention time of 3min. Stability of bacteriocin was nil when treated with protease K, whereas lysozyme, pepsin and α-amylase has enchanced the activity. Bacteriocin was stable at temperature from 20-110p C and pH 2.0 –pH8.0, stability with detergents and surfactant such as Triton X-100, SDS, Tween-80 showed no change in stability whereas stability was more with Nacl. This work shows that the bacteriocin from *L. plantarum* is stable between temp 20-110p C and pH 2.0-pH 8.0, thus can be used to control the growth of harmful bacteria in a variety of food products, dairy products, meat products, and vegetables.

Key words : Antibiotic resistance, Antimicrobial peptide, Neera, probiotic drink.

Growing issue of Multi Drug Resistant (MDR) pathogens and rapidly declining antibiotic arsenal may be biggest problems facing humanity in the 21st century. Drug resistance is one of the most serious health threats facing humanity. It could cause 10 million deaths per year[22]. One alternative is the use of bacteriocins, which are antimicrobial peptides produced by bacteria which kill or inhibit the growth of other bacteria, including those that are pathogens[1]. Bacteriocins are typically small in size and have a broad spectrum of activity against bacteria. Bacteriocins are considered as promising alternatives to traditional antibiotics, as they are less likely to cause antibiotic resistance and have fewer side effects[1]. Bacteriocins are potential alternatives to antibiotics in management of MDR infections[4]. Emergence of MDR pathogens has reduced the efficacy of common antibiotics, and as a result, there is a particular need for development of new antimicrobial agents[4]. Bacteriocins are considered potential alternatives to traditional antibiotics for combating MDR pathogens. Bacteriocins are classified as Class I: Lantibiotics - small, heat-stable peptides that contain unusual amino acids, such as lanthionine and methyllanthionine, which are formed by post-translational modifications. Class II: Non-Lantibiotics - larger, heat-labile peptides that do not contain lanthionine or methyllanthionine, but have disulfide bridges, β-sheets or α-helices structures. Class III: Large heat-labile proteins with diverse structures and mechanisms of action, such as bacteriophages, toxins and bacteriocins produced by *Bacillus species*. Class IV: Complex bacteriocins -heterogenous class that contains different types of bacteriocins, such as two-peptide bacteriocins, circular bacteriocins, bacteriocins with lipid II binding domains[11]. Bacteriocin have various applications food preservation and also been reported to have various therapeutic purposes, including treatment of peptic ulcers, as a spermicidal agent, for woman care, as an anti-cancerous agent, and for plant growth promotion in agriculture[1].

Isolation and Identification of LAB :

LAB was isolated from Neera (a probiotic drink)[17]. Fresh neera was collected from the Karnataka border, of Telangana state Hyderabad. 1ml of each sample was cultured on De Man Rogosa and Sharpe agar (MRS), plate for 24h at 37°C. Selected colonies with distinct morphology were subcultured on MRS agar plates. Only catalase-negative, Gram positive colonies were assumed to be LAB.

Detection of Antimicrobial Activity of Isolated Bacterial strain :

Detect the antimicrobial activity of these isolated LAB was done by well diffusion assay methods.LAB isolate grown in MRS broth at 37°C for 48h under stationary condition, fermented broth was centrifuged at 10,000 rpm for 10 min. Antimicrobial activity of supernatant was tested by agar well diffusion method. An overnight culture of test organisms grown in their respective medium at 37°C was diluted to a turbidity equivalent to that of a 0.5 McFarland standard[6].

Primary Characterization and Molecular Identification of VRN-25 :

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[9]. 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)[2] and sequence was submitted to GenBank sequence database and accession number was obtained.

Purification of Bacteriocin from Lactiplantibacillus plantarum strain 1625:

For the bacteriocin purification, supernatant was collected after extraction was done by 70% ammonium sulfate precipitation incubated overnight at 4°C and was again centrifuged at 15,000rpm for 10min.The centrifuged broth was collected and dialyzed extracted by solvent method using Cholorform: Methanol (2:1)[5]. Purity of bacteriocin was determined by HPLC equipped with a chromatographic column of SB-C18. Bacteriocin was loaded into the HPLC column which contains Solvent A (Water: Acteonitrile = 1:99) 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. One ml samples was monitored at 280nm[16].

Effects of Enzymes, pH, Temperature Detergents and surfactants on Bacteriocins:

Partially purified bacteriocin was incubated for 2h at 37°C with 1mg/ml of enzymes trypsin , proteinase-K, α-amylase, pepsin, cellulose, and lysozymes, the antimicrobial activity of bacteriocin was checked. Partially purified bacteriocin with pH 2.0-11.0 with sterile 1M NaoH and 1M Hcl were incubated for 2h and antimicrobial activity of bacteriocin was checked. Effect of temperature on Bacteriocins activity was evaluated by heating at 20-121°C. Partially purified bacteriocin was incubated at 37°C for 2h with 1 % (w/v) of SDS, Tween-20, Tween-80, Triton X-100, Nacl, EDTA, β-mercaptoethanol and the antimicrobial activity of sample was checked by agar well diffusion assay[20].

Isolation of Bacterioicin producing strain and its antimicrobial activity :

A total of 45 Gram positive and catalase negative LAB strains were isolated from traditional probiotic drink *Neera*, of which 40 LAB strains exhibited antimicrobial activity against *Escherichia coli(ATCC-8739), Pseudomonas aeruginosa (ATCC-9027), Proteus vulagaris, S. epidermidis (ATCC-12228), S. aureus(ATCC-6538), P. plecoglossicida, Bacillus subtilis (ATCC-6633), Streptococcus faecalis (ATCC-8043), Candida albicans (ATCC-10231), C. glabrata, C. haemulone* by disc diffusion method.

Identification of VRN-25 as Lactiplantibacillus plantarum strain 1625
Isolate VRN-25 Isolated from neera (probiotic drink) was characterized as gram positive, non-flagellated, nonspore forming, rod shaped

bacterium that doesn't show catalase activity (Table-1). Isolated strain was next identified and confirmed as *Lactiplantibacillus plantarum 1625* (Accession no-ON692894). Its neighbour joining phylogenetic tree was constructed by sequence alignment comparison using MEGA-7 software. Analysis of strain's 16sRNA nucleotide sequence revealed 100% similarity with that of *Lactobacillus plantarum* strain 1625 and *Lactobacillus plantarum* strain 225(Table-2). Several *L. plantarum* strains have been isolated from many fermented foods and vegetables, including *L. plantarum* KLDS1.0391[7], *L. plantarum* LB-B1[22], *L. plantarum* LPCO10[10], *L. plantarum* C19[3], *L. plantarum* KLAB21[15], *and L. plantarum* 163 [13], which were isolated from different fermented cream, fermented foods and traditional Chinese fermented vegetables respectively. In the present study strain VRN-25 was identified as *Lactiplantibacilli-plantarum* strain 1625 (ON692894) which is isolated from neera a traditional probiotic drink. Therefore, findings suggest that *L. plantarum* is isolated from neera may have particularly high bacteriocins production (Fig. 1a & b) capability.

Antimicrobial spectrum Bacteriocin producing L. plantarum :

Partially purified bacteriocins produced by *L. plantarum* was purified by ammonium sulphate precipitation exhibited a good antimicrobial spectrum (Table-3) and significantly inhibited gram negative *Escherichia coli (ATCC-8739), Klebsiella pneumoniae (ATCC-2706), Pseudomonas aeruginosa (ATCC-9027), Proteus vulgaris, P. plecoglossicida,* and Gram positive *S. epidermidis (ATCC-9027), S.aureus (6538), Streptococcus faecalis* (8043)*, B. subtilis (ATCC6633),* and fungal pathogens *Candida albicans (ATCC-10231), C. glabrata, C. haemulone.* In our study *K. pneumoniae* has shown zone of inhibition of 27mm and *S. aureus* (6538) showed 22mm zone of inhibition, while *C. glabrata* showed 16mm zone of inhibition (Table-3: Fig. 2 a,b). Plantaricins produced by *L. plantarum* strain has shown zone of 4mm with *L. monocytogenes (ATCC-1911) and 3mm with S.aureus D-5*[23]. Plantaricin produced by *L. plantarum LP31* showed bactericidial activity against *Pseudomonas* sp, *Staphylococcus aureus, Bacillus cereus, and Listeria monocytogenes* with 15-20mm zone of inhibition[14] Similarly some of the Bacteriocins found to have similar activity produced by Plantaricin 163 showed inhibition zone against gram positive and gram negative bacteria[15]. Planticin MG has showed inhibitory activity against *L. monocytogenes, S. aureus, S. typhimurim, and E. coli*[7].

Purification of Bacteriocin by L. plantarum strain 1625 *:*

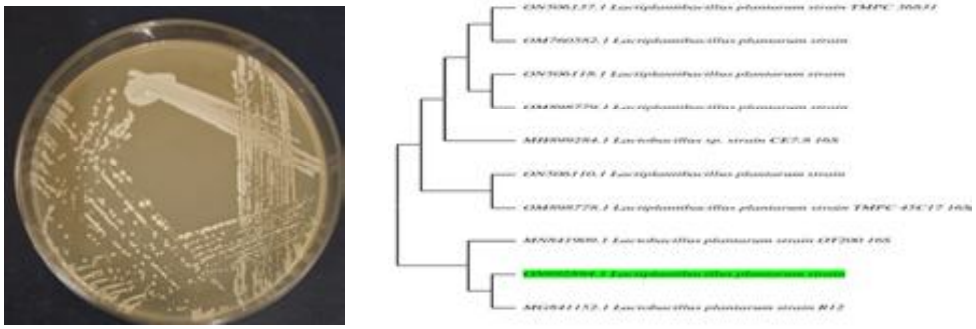
Partially purified bacteriocin from *L. plantarum* were obtained by chromatography. Partially purified fraction revealed a maximum absorbance at 280nm. Following final purification by RP-HPLC, the chromatogram showed only one peak, where the retention time was 3min (Fig. 3). Our results correlate with Ranga *et.al.*,[18] the purified sample showed a distinct peak at 2.192 min corresponding to the peak at 2.192 min for standard bacteriocin. Some studies have employed these techniques for purification of plantaricin 163[13] Plantaricin MG[7], Plantaricin GZ1-27[8], Plantaricin LR14α[19].

Table -1 *Biochemical Characteristics of the Isolate*-VRN-25

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

Table-2 *Genomic Identification of Identified Bacterial species*-VRN-25

BLAST results	Gen bank accession number
Lactiplantibacillus plantarum strain 1625	ON692894



a) b)

Fig. 1a Purified colonies of *Lactiplantibacillus plantarum* strain on MRS
b. Phylogenetic tree of *Lactiplantibacillus plantarum* strain 162

Table-3 Zone Of Inhibition By Bacteriocin Producing *Lactiplantibacillus Plantarum Strain* 1625

Test organsism	Zone of inhibition
Escherichia coli(ATCC-8739)	16mm
Pseudomenas aeruginosa(ATCC-9027)	12mm
Klebsiella pneumonia	27mm
Proteus vulgaris	20mm
Slaphylococcus aureus(6538)	22mm
Slaphylococcus epidermidis(ATCC-12228)	15mm
Streptoccocus faecalis(8043)	15mm
Pseudomonas plecoglossicida	15mm
Listeria monocytogenes	12mm
Bacillus subtilius	21mm
Candida haemulonei.	15mm
Candida glabrata, *Candida albicans(ATCC-10231)*	16mm 14mm

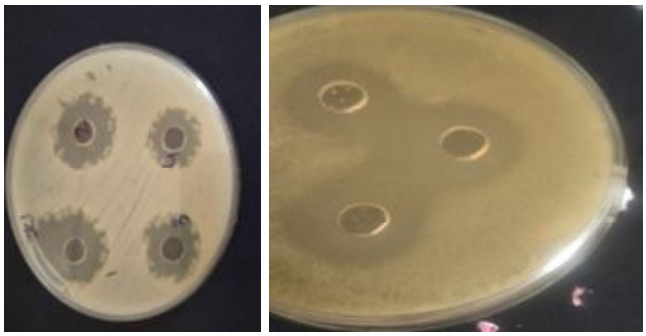


Fig. 2. Zone of Growth Inhibition against pathogen by bacteriocin produced by *L. plantarum strain* 1625

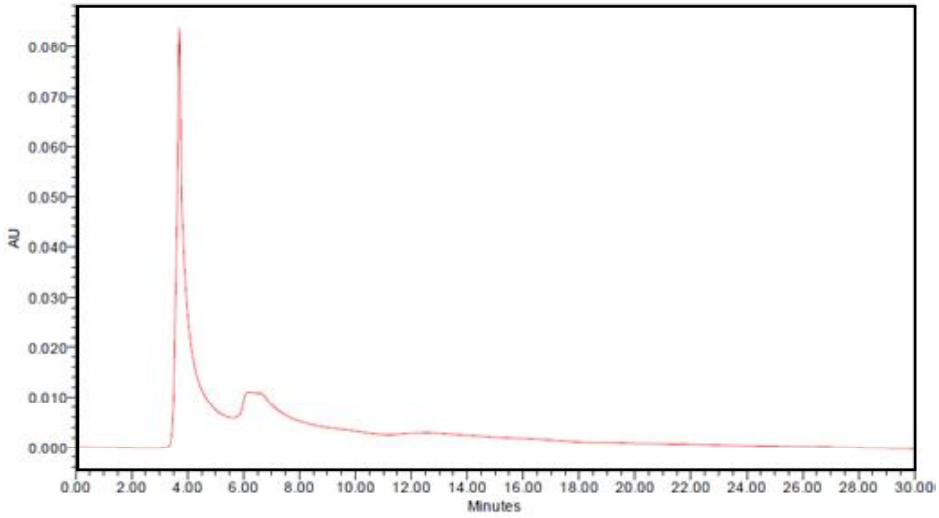


Fig. 3. HPLC peak at retention time of 3min

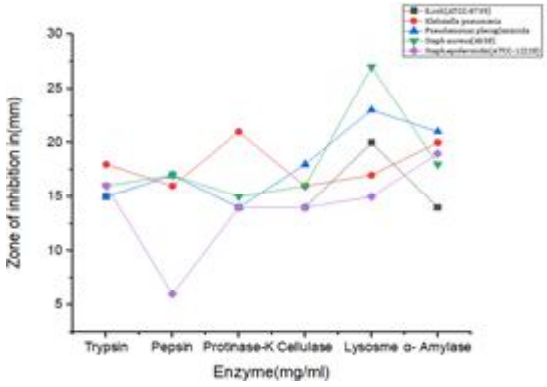


Fig. 4. Effects of Enzymes

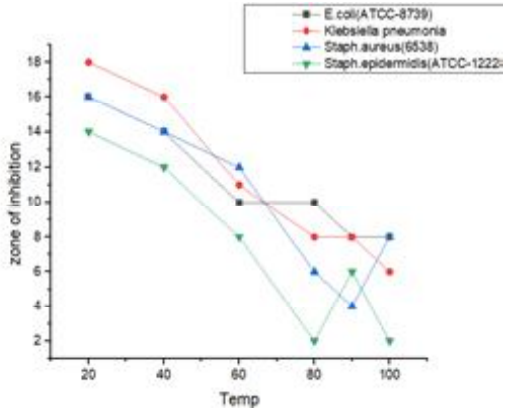


Fig. 5. Effects of Temperature

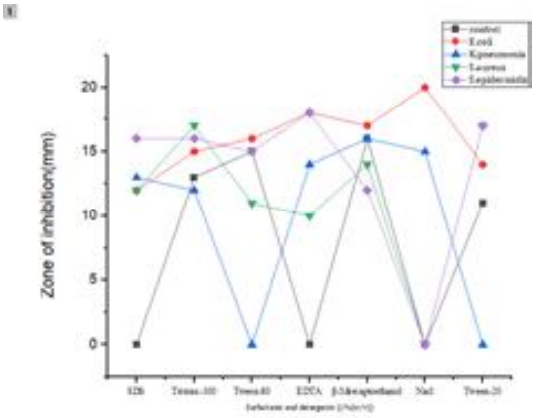


Fig. 6. Effects of pH

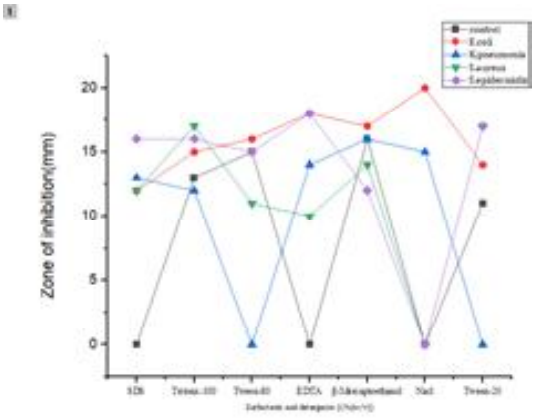


Fig. 7. Effects of Surfantant and Detergent

Effect of Enzyme, Temperature, and pH, Surfactant and Detergent Effect on Bacteriocins Produced by L. platarum:

Stability of bacteriocin produced by *L. plantarum* was tested against *E. coli* (ATCC-8739), *P. aeruginosa* (*ATCC9027), K. pneumoniae (ATCC2706), S. aureus (6538), S. epidermidis (ATCC-12228)*. Antimicrobial activity of *L. plantarum* was nil after treatment with protease-K, whereas Lysozyme, pepsin and α-amylase has enhanced the activity, trypsin and cellulose has reduced the activity (Fig. 4). Bacteriocin produced by *L. plantarum* was tested at different temperatures (20, 40,60,80,100,110,121), (Fig. 5) and pH from pH 2.0 to pH 12.0 at which the bacteriocin was stable at temperature from 20-110°C, where as the stability was lost at 121°C and pH stability of bacteriocin was from pH 2.0 to 8.0, whereas the stability was lost after the pH 9.0 (Fig. 6). Bacteriocin stability with detergent and surfactant was checked with SDS, Tween-20, tween-80, TritonX-100, Nacl, EDTA, β-mercaptoethanol, which showed no change in activity with Triton X-100, SDS, Tween-80, whereas with Nacl the stability was more (Fig. 7). Our results correlate with *Lactobacillus plantarum* 163 which produce plactaricin was stable at 60,80,100,121°C and pH from pH 2.0 to pH 10.0, and the enzyme stability was lost with protease K[13] and temperature and pH the bacteriocin was stable at 60,80,100,121°C and pH from 2.0-10.0 and also the stability of Bacteriocins stable after treatment with organic solvents, surfactants, and detergents but increased in response to EDTA when compared with our result[12]. Our results revealed no response to EDTA but Nacl enhance the zone of inhibition.

In the study bacteriocin was isolated from *L. plantarum* which has shown zone of inhibition against gram positive and gram negative and fungal pathogen which are active at small amount and they are species specific which shows that, these bacteriocins can be used in medical fields as an alternative for antibiotic. Bacteriocin produced by the *L. plantarum* is stable at high temperature and low pH which can be used in food preservation.

References :

1. Abebe Worku Negash and Berhanu Andualem Tsehai (2020). *International Journal of Microbiology*, Article ID 4374891, 7 pages https://doi.org/10.1155/2020/4374891.
2. Altschul Stephen, F., L. Thomas, A. Madden, Alejandro Schaffer, Jinghui Zhang, Zheng Zhang, Webb, David, Miller and J. Lipman (1997) *Nucleic Acids Res*., *25:* 3389-3402.
3. Atrih, A., N. Rekhif, J. Milliere, and G. Lefebvre, (1993) *Can. J. Microbiol, 39:* 1173-1179.
4. Benítez-Chao D.F., A. León-Buitimea, J.A. Lerma-Escalera and J.R. Morones-Ramírez (2021) *Front. Microbiol. 12:* 630695 doi: 10.3389/fmicb.2021.630695.
5. Deshmukh P.V. and P.R. Thorat (2013). WHL 3, *Int. J. Pharm. Sci. Rev. Res. 22*(2): 180-185.
6. Emmanuel E.S. Challaraj*, Apoorva Udayashankar and Jyoti Mishra (2018) *JPAM* https://dx.doi.org/10.22207/JPAM.11.2.45.
7. Gong, H.S., X. C. Meng, and H. Wang, (2010) Plantaricin MG active against Gram-negative bacteria produced by Lactobacillus plantarum KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China. *Food Control 21:* 89-96.
8. Hechao Du , Jie Yang, Xiaohong Lu, Zhaoxin Lu, Xiaomei Bie, Haizhen Zhao, Chong Zhang, Fengxia LuPurification, Characterization, and Mode of Action of Plantaricin GZ1-27, a Novel Bacteriocin against Bacillus cereus J Agric Food Chem. 2018 May 9;66(18):4716-4724. doi: 10.1021/acs.jafc.8b01124. Epub 2018 May 1.
9. Holt, J.G., N.R. Kreig, P.H.A., Sneath, J.T., Staley, and S.T. Williams. (1994) *Bergey's manual of determinative bacteriology Williams and Wilkins, Maryland, USA.,* 9th Edn.
10. Jimenez-Díaz, R., R. Rios-Sanchez, M. Desmazeaud, J. L. Ruiz- ì Barba, and J.C. Piard, (1993) Plantaricins S *Appl. Environ. Microbiol., 59:* 1416-1424.
11. Klaenhammer, T. R. (1993). *Journal of dairy science, 76*(9): 2485-2491. doi:10.3168/jds.S0022-0302(93)776122.
12. Li-Li Man, and Dian-Jun Xiang *Folia Microbiologica* https://doi.org/10.1007/s12223-019-00697-0.
13. Meizhong Hu, Haizhen Zhao, Chong Zhang, Jiansheng Yu, and Lu Zhaoxin (2013) dx.doi.org/10.1021/jf403370y | *J. Agric. Food Chem., 61:* 11676-11682.
14. Müller, D.M.[1], M.S. Carrasco, G.G. Tonarelli, and A. C. Simonetta (2009) *J. Appl Microbiol Jun;106*(6): 2031-40. doi: 10.1111/j.1365-2672.2009.04173.x. Epub 2009 Mar 19.
15. Park, H.D. and C.H. Rhee, (2001) *Biotechnol. Lett., 23:* 1583-1589.
16. Pingitore, E. Vera, E. Salvucci, F. Sesma, and M. E. Nader-Macías* Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB) Communicating *Current Research and Educational Topics and Trends in Applied Microbiology*
17. Poornachandra Rao K., B. V. Deepthi, S. Rakesh, T. Ganesh, P. Achar and M.Y. Sreenivasa (2017). *Probiotics Antimicrob.*

Proteins 11: 55–64. 10.1007/s12602-017-9338-y

18. RangaMounika, Jannatul F. Siddique, Nivetha Anbalagan, Subathra C. Devi and V. Mohanasrinivasan (2021) Purification of Bacteriocin from Lactobacillus plantarum KY449289 and its Role in the Preservation of Mixed Fruit Juice Volume 17, Issue 7, 2021Article ID: e010621187202 Pages: 8 DOI: 10.2174/1573407216999201026193645.
19. Tiwari Santosh Kumar and Sheela Srivastava (2015) *Austin J Anal Pharm Chem., 2*(2): 1036.
20. Todorov S.D., and L.M.T. Dick, (2007) *Brazilian Journal of Microbiology, 38:* 166-172.
21. WHO. (2020). Antimicrobial resistance. World Health Organization.
22. Xie, Y., H. An, Y. Hao, Q. Qin, Y. Huang, Y. Luo, and L. Zhang, Characterization of an anti-Listeria bacteriocin produced by Lactobacillus plantarum LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. Food Co