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Isolation, Characterization of *Lactobacillus Delbrueckii*derived Bacteriocin and Assessment of its Bioactive Potential

Vijaya Rayapu[1],K. Revathi[2*], A. Arunagirinathan[3], SenthilPragash D[4],S. Khaja Mohiddin[5]
[1]ReseachScholor, MeenakshiAcademy Of Higher Education And Research, Chennai.
[2]Research Director, Meenakshi Academy Of Higher Education And Research,Chennai
[3]Academic Director, Meenakshi Academy Of Higher Education And Research,Chennai.
[4] Microbiologist, Central Leprosy Teaching And Research Institute,Chengalpattu.
[5] Assistant Professor, Government Medical College And Hospital ,Kadapa, Andhra Pradesh.

ABSTRACT

A Lactobacillus delbrueckii strain that produces bacteriocin was isolated from the vagina of a healthy person. A multitude of bacterial species, including Pseudomonas, Staphylococcus, Salmonella, and Enterococcus, were inhibited by the supernatant fluid. Bacteriocin is an antimicrobial peptide which was isolated from Lactobacillus delbrueckii. A single four-step process was carried out to purify it to homogeneity. MALDI-TOF investigation was performed with the crude supernatant acquired from an initial stationary phase culture in the MRS broth. Bacteriocin was proteolytic enzyme sensitive, heat resistant, functional over a broad pH range, and drifted as a peptide of 4.5–5.0. kDain the SDS–PAGE. During exponential growth, the bacteriocin was formed continuously. It was bactericidal to sensitive cells, and cell lysis did not achieve the bactericidal impact. The bacteriocin's amino acid composition was measured, and no modified amino acids were discovered among the residues found.

Keywords:
Lactobacillus delbrueckii, MALDI-TOF analysis, Bacteriocin

Introduction

Bacteriocins are peptides or proteins that are made by ribosome which has medicament activity against strains from various species or genera (De Vuyst and Leroy, 2007). However, a growing variety of innovative bacteriocins towards foodborne pathogens are well-known (Liu et al., 2015; Yi et al., 2016; Zhao et al., 2016). Bacteriocins developed by lactic acid producing bacterial communities (LAB) have recently received loads of attention because of their potential as safe and economical bio-preservative agents. It's conjointly documented that bacteriocins are proficient of constraining the progression of foodborne pathogens such as*Staphylococcusaureus*, *Escherichia coli*, and*Listeriamonocytogenes*.

Bacteriocins are normally used for natural food preservation and protection in several countries also as being safe for humans (Liu et al., 2016, 2017; Yi et al., 2016, Cotter et al., 2013; principle et al., 2014). Since its 1st exploitation in England in 1953, the bacteriocinnisin that was discovered in New Island in 1933 has been approved to be used in forty eight countries (Tagg et al., 1995). Bacteriocins are often classified into 3 main clubsbased on their structural, physicochemical, and molecular properties. Lantibiotics are small category 1 bacteriocins, i.e. cationic, heat-stable amides, hydro-phobic, and that comprise uncommon amino acids that are synthesized during the post-translation modification. Apart from the cleavage of a frontrunner peptide from the pre-bacteriocin peptide, category two bacteriocins are small, cationic, hydrophobic, heat-stable peptides that don't seem to be post-translationallymodified.There are three subclasses beneath this group: Bacteriocins belonging to 2asubclass, or pediocin-like bacteriocins, with an impact on food borne pathogenic communities and the consent sequence Tyr-Gly-Asn-Gly-Val in N-terminusdomine; Subclass 2b, which are bacteriocins which need dual polypeptide chains forthe maximum action, and Subclass 2c, are the bacteriocins which is

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not belonging to subgroups. Bacteriocins of class 3 is a bulky,thermo-labile, and hydrophilicmolecules.Therefore in the present investigation, an effort was taken to develop, refine, and characterize bacteriocin from *Lactobacillus delbruckii*, a human vaginal bacterium. Purified bacteriocin was also put to the test for antimicrobial activity against possible pathogens. Other bioactivities, apart from antimicrobial activity, have been extensively examined. Purified bacteriocin was also put to the test for antimicrobial activity towards possible pathogenic microorganisms. Other bioactivities, in addition to antimicrobial potential, have been extensively examined.

MATERIAL AND METHODS

Bacterial Strains used andits culture conditions: Serial dilution and plating technique in MRS medium was performed to evaluate the growthof*L. delbrueckii*.The*L. delbrueckii* strains were cultured andmaintained in theliquid of MRS complex medium (Difco). For the sub culturing, fresh culture cells with the optical density measurementof 0.1 at 600 nm (OD_{600}) that were first washed in 0.9% NaCl for the inoculation of liquid cultures in MRS Broth.

Determination of organic acids by *L. delbrueckii*andacidity:The acid produced by *L. delbrueckii* was tested for quality and quantity.According to previous studies, the quantities of organic acids released by *L. delbrueckii* were measured by the difference between the volume of organic acid in the supernatants earlier and later of the fermentation process.Each acid's concentrations were measured in millimoles per liter.The experiment was performed in triplicates to conclude the final values. Cells (or cell-free excerpt) from L. delbrueckii was resuspended in the cold phosphate buffer with the molarity of 0.2 M and with a corresponding pH 6.5 with or without 55.5 mM glucose and incubated at 5°C for the definedperiod. After the incubation period, the cells were centrifuged to be quantified.

Hydrogen peroxide production by *L. delbrueckii*:*L. delbrueckii's*cell-free supernatant (5 mL) from cells were combined with 1 mL of 0.1 per cent aqueous peroxidase (Horseradish Type VI-A; Sigma Chemical) following that 0.1 mL of 1 % of o-dianisidine aqueous solution was added.As a blank, phosphate buffer (5 mL)was used as an alternative of the experiment sample.The tubes were then further incubated at 37°Cfor 10 min. To stop the reaction, 0.5 mL of 2N HCl was added to the tubes. The optical density at 400 nm (OD_{400} nm) of the test samples wereassessed, and the peroxide quantity wasmeasured by comparing the OD_{400} nm absorbance with astandardplot(Gilliland, 1969).

Synthesis of crude Bacteriocin: In MRS broth, *L. delbrueckii* was cultured aerobically for 24 hours at 35°C (Hi-Media, India; pH 6.5). To remove the cells from the growth media, they were centrifuged in the following condition 8,000 rpmfor 30 minat 4 °Cand separated with 0.22 µ filters after the incubation period.Thesupernatant'spH was modified using 1N NaOH to6.0. The resultant was used as crude bacteriocin.

Assessment of BacteriocinActivity: The agar well diffusion assay was performed to assess bacteriocinactivity.*L. delbrueckii*was seeded on Mueller-Hinton agar plates. Wells with a diameter of 6 mm were drilled, and aliquots (20 µL) of the supernatant were inoculatedinto the wells and kept for 24 hours.The diameter of growth reticence was evaluated after incubation period of 24h. The arbitrary units per milliliter (AU/ml) were used to calculate the activity of the cell-free supernatant.Thebacteriocin's unit activity was defined as 1 AU, which is equal to a unit area of zoneofinhibiton per unit volume, in this case 2 mm/ml (Usmiati&Marwati 2009).The assay wasperformed as replicate 3 times.Thebacteriocin activity was calculated according to the standard formula.

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Partial characterization of bacteriocin:*L. delbrueckii* was cultured in MRS broth for 24 to 48 h at 37 °C before being partly characterized. The grown cells were centrifuged at 8,000 rpm for 1 h at 4°C after incubation.The precipitation ofBacteriocins was done with ammonium sulfate (80 per cent saturation) andkept overnight at-20 °C for augmented precipitation. The entire mixture was centrifuged after precipitation and pellets were obtained and stored in a potassium phosphate buffer with a pH of 7.2.The precipitate was dialyzed at 4°C for 12 hours against a 20 mM potassium phosphate buffer (pH 7.0). (2010 by Rajaram et al).

Influence on BacteriocinActivitybyTemperature: Various heat treatments were used for dialyzed bacteriocin (pH 5.5, 500 l): 30°C to 100°C for 30 min and 121°C for 15 min. *Bacillus cereus* was used as an indicator organism to evaluate the activity of bacteriocin.

Influence on BacteriocinActivityby pH: The effect of pH was investigated using bacteriocin (pH 5.5, 50 l) mixed with 100 l of buffer (0.1M potassium phosphate pH 4to pH9) and incubated at 60°C for 1 hour before determining bacteriocin activity against Bacillus cereus.

Influence on Bacteriocin Activity by detergents:sodium dodecyl sulfate,Tween 80, and Triton-X100surfactants were applied to the bacteriocin solutions in concentrations of 100 µL or 0.01 g/ml (pH 5.5). Bacteriocin activity against Bacillus cereus was determined by incubating these preparations at 60°C for 60 minutes.

Influence on Bacteriocin Activity by enzymes: On bacteriocin preparations, the enzymes D-amylase, lysozyme, protease, and proteinase-K were each measured at a final concentration of 1 mg/ml (pH 5.5). It was then incubated
For 2 hours at 60°C to assess bacteriocin activity against the pathogens.

Assessment of Bacteriocinactivity: The spot-on-lawn approach was used to measure arbitrary units (AU) of bacteriocin activities (Yamamoto et al. 2003).The previously mentioned method was used to obtain cell free supernatant. Filter sterilized 20 mMpotassium phosphate buffer (pH 6.5) was used to dilute the resulting sample twofold.After applying 100 μl of *L. delbrueckii* overnight culture, soft agar (20 ml) was solidified in a sterile petri dish.After 30 minutes of drying, 10 μl of each diluted sample was spotted onto the plate and incubated at 37°C overnight.The titter was measured as the reciprocal of the highest dilution (2n) that caused the indicator lawn to be inhibited. In consequently, the antibacterial activity per mL was defined in AU as $2^n \times 1000 \ \mu L \ /10 \ \mu l^{-1}$.

BacteriocinPassive Elution from Polyacrylamide Gel: *Lactobacillus delbrueckii*crude proteins were fractionated using the Schagger and Von Jagow method of Tricine-SDS-PAGE (16 percent polyacrylamide gel).The gels were stained with Coomassie brilliant blue after electrophoresis was completed. The gel was eroded three times in 0.1 percent Tween 80 at 37°C to eliminate SDS and spotthebandsof protein with antibacterial activity.Then, using Martinez et al process, soft agar medium (10 ml) with test pathogens (approximately $1* 10^6$ to $2*10^6$ cells/ml) was shrouded to overlay the entire gel (6 by 9 cm). Subsequently overnight incubation at 30°C, clear zones around the protein bands were found, suggesting that the protein has antibacterial activity.

BacteriocinIdentification by Mass Spectrometry: The entire mass spectraof the bacteriocin was identified using a rectilinear MALDI-TOF mass spectrometer with delayed extraction technology with a 125-cm flight tube For desorption/ionization of the samples, whole spectra were acquired in positive ion linear mode with a nitrogen laser (l 5 337 nm) and a 20 kV acceleration voltage.The spectra are the product of a sequence of 60 laser shots. Angiotensin II and insulin bovine were used for external mass calibration (MH1 5 5,734.557; Sigma). For each sample preparation phase, the instrument was calibrated.

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Bioactive potential of bacteriocin isolated

***In vitro* fungicidal effect of *L. delbrueckii*bacteriocin:**At five and 20°C, the performance of L. delbrueckiibacteriocin in opposition to fungal pathogens became evaluated the use of the microdilutionapproachdescribedthroughLavermicocca et al. (2003) with a few modifications. At 80°C, the inventory cultures had beensaved as spore or mycelial suspensions in 15% glycerol (v/v). The fungi's conidia had beenaccumulated from four day-vintage cultures grown at 25°C on potato dextrose agar (PDA). The fungi's conidia had beenaccumulated from 12-day-vintage cultures grown on Pseudomonas Agar F at 22°C below a 12 h light/darkish cycle. The fungi's conidia had beenaccumulated from 12-day-vintage cultures grown at 25°C on changed V-8TM medium. Using sterile distilled water, the density of spore suspensions became diluted to twoone zero five spores ml-1, as calculated through a hemocytometer. In sterile 96-nicelymicrodilution plates, microdilutioncheckshadbeenperformed.Themicrodilution plates had been incubated at both 20 and5°C after two hundred l of take a look atanswer containing 185 l of L. delbrueckiibacteriocin and 15 l of conidial suspension becamedisbursed into the wells. Using a microtiter plate reader/spectrophotometer, the optical density (OD) became calculated at 580 nm in specific time intervals (Spectra MAX 190, Molecular Devices, CA, USA). The OD of control plates incubated at 20°C became measured at 0, 24, 40, 48, or seventy two hours, at the same time as the OD of tradition plates incubated at five°Cbecame measured at 0, 48, seventy two, 96, or one hundred twenty hours. The testbecame replicated 3 times. (185 l MRS broth and 15 l conidial suspension) and a poor control (185 l LAB CFS and 15 l lifeless conidial suspension) had beenorganized and maintained for every fungus. The testbecame replicated 3 times.

Cell culture : HepG2.2.15 cells were grown in RPMI 1640 complemented by 10% fetal bovine serum and 1% (v/v) penicillin (10,000 U/ml)/streptomycin (10,000 U/ml) under 5% CO2 at 37°C.Trypsin therapy was used to remove sub-confluent monolayer cells from culture dishes, which were then transferred to fresh cell culture dishes. The blue dye exclusion system of trypan was used to estimate cell number (Strober 2001).

Anti-cancer activity of bacteriocin: Five separate human cancer cell lines, including HeLa, MCF-7, HT1080, H1299, and HEK293T, were used to assess the anticancer efficacy of L. delbrueckiibacteriocin.The cell lines were incubated with increasing concentrations of bacteriocins (1–20 M) in the specific growth medium, revealing that LS10 has cytotoxic efficacy against all five cancer cell lines in nature.Whole cancer cell lines showed cytotoxicity based on the dose of bacteriosin; highest effect was observed at 10 μM, and the highest activity was observed against the MCF-7 cell line.Although MCF-7 showed 40% cytotoxicity at a 5 μM concentration of LS10, HT1080, HEK293T, and H1299 showed just 20% cytotoxicity.However, at a concentration of 5 μM, no major cytotoxicity was observed in HeLa cells, but at a concentration of 10 μM, cytotoxicity was observed in 80 percent of HeLa and other cell lines.

Results and Discussion

Measurement of organic acids production and acidity by *L. delbrueckii*:The results revealed that the complex MRS broth medium yielded the most lactic acid (300–550mmol l-1).It's apparent because of the media's ideal composition, which was designed especially for the *Lactobacillus bacterium*glucose is the major carbohydrate source in the mediafermented two pathways by*L. delbrueckii.*

Lactic acid is the major product when glucose is used as the main substratein the metabolic pathways shown by our findings. Adding to that, glycolysis generates 2 mol of lactic acid is formed from 1 mol of glucose, while the 6-PG/PK pathway produces just 1 mole of lactic acid from 1 mol of glucose. Based on our findings from this study, the strain *L. delbrueckii* appears to

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be hetero-fermentative in several conditions, but certain other factors can play a role. Figure 1 the result shows the quantification of lactic acid produced by the isolates. *L. acidophilus* yielded the huge amount of lactic acid after 24 hours, according to the results.

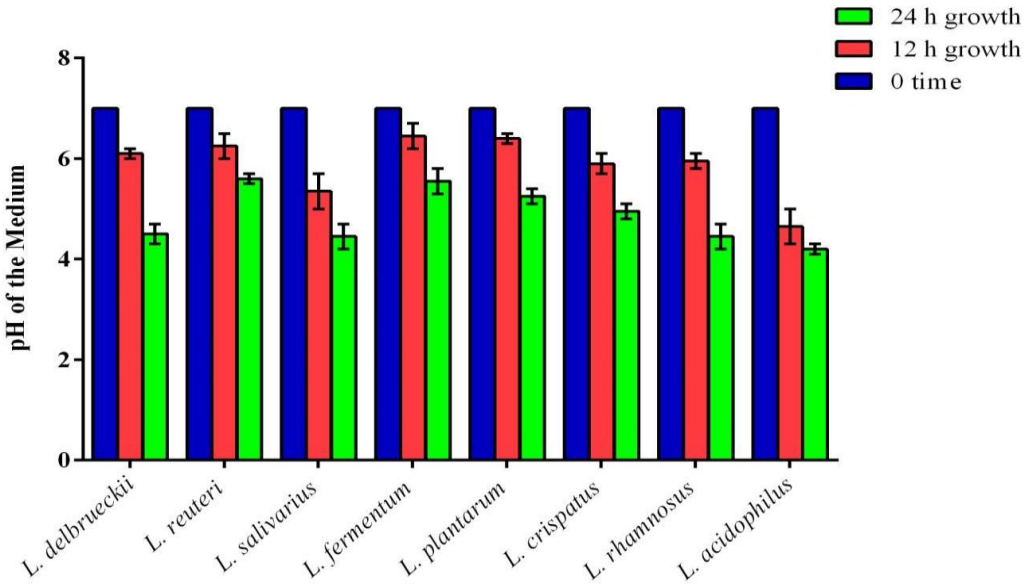

Figure 1 –production of organic acid by the isolates in the 24 h of incubation

H_2O_2 production by the isolate: Hydrogen peroxide, an antagonistic chemical, is also used in lactobacilli. Quantitative approaches are typically used to assess the performance. In the current study, the isolate L. delbrueckii produced the highest amount of H_2O_2, 3.18 mg/ml. According to the findings, lactic acid production improved as the incubation time increased from 24 to 72 hours.

Determination of bacteriocin activity: Since During the mid phase, the highest activity was found, and the bacteriocin was isolated from Lactobacillus delbrueckii culture during this period. The secreted bacteriocin's highest antimicrobial activity was strain-specific, ranging from 350 to 520 AU/ml. When trypsin loses its bactericidal function, it indicates that the activity is attributed to a protein. As a result, using commercial nisin as a reference, the bacteriocin activity was measured in Arbitory Units/ml (AU/ml). Figure 3.2 depicts the normal graph for nisinbacteriocinogenic activity. The only disadvantage is that, because of its hydrophobic nature, nisin diffusion inside agar media is only corresponding to its concentration over a narrow linear array (Chandrapati et al., 1998). The non-linear norm chart, on the other hand, is also useful for assessing other bacteriocinogenic behavior.

Sensitivity of bacteriocin to pH, enzymes and temperature: When incubated for 15 and 30 minutes at 60, 80, and 100 degrees Celsius, no substantial variations in antimicrobial activity were observed (P 0.05). (Table 4.23).Bacteriocin were stable at 37°C with pH of 2.0 to 10.0 after incubation (P0.05) showed that bacteriocin be able to treat both acidic and non-acidic foods. Bacteriocin's antimicrobial efficacy was not inactivated at high temperatures, such as boiling (60°C, 30 minutes) or boiling (100°C, 45 minutes). Catalase therapy did not result in a loss of bacteriocin function, suggesting that H2O2 was not the source of reticence activity. Bacteriocin treatment with enzymes like proteinase K and pepsin forms a absolute loss of bacteriocin activity, suggesting that the effective portion of bacteriocin was protein in nature, while non-treated bacteriocin had a substantial (P0.01) bacteriocin activity. The bacteriocin action was slightly

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reduced by the presence of -amylase, DNase, and RNase. These findings indicate that the biocontrol agent was protein in nature and that its action was solely based on glycosylation, implying that it was active in cooperation with the glycol and protein portions. Sodium dodecyl sulphate (SDS), Tween 80, EDTA, Tween 20, and urea were used as detergents. Tween 80, SDS, and Tween 20 have no effect on bacteriocin activity, which means that these detergents have no effect on bacteriocin activity. The bacteriocin activity was greatly decreased when the assay was done after treatment (P0.05). (See Figure).

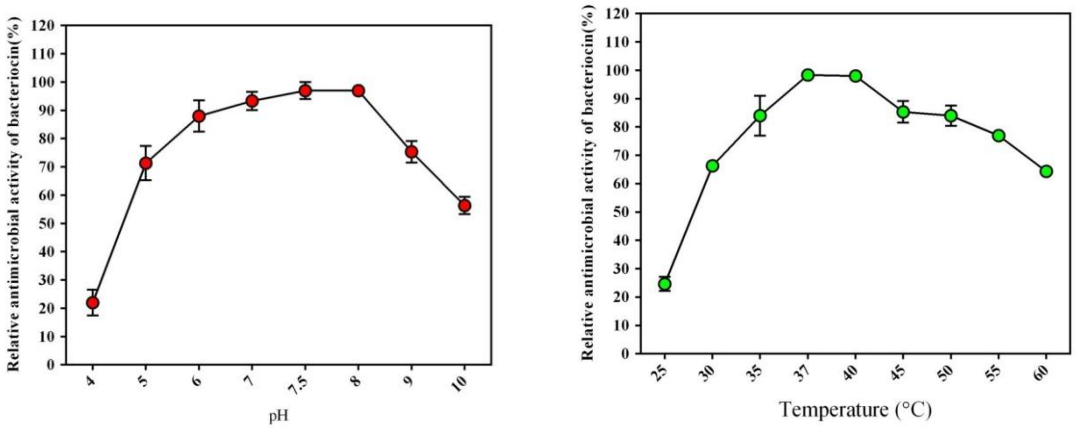

Figure 2 A- Stability of bacteriocin at various pH Figure 2 B - Temperature stability of the bacteriocin

The lack of bacteriocin activity reduction or loss after detergent treatment may be attributed to non-denaturation of the bacteriocin's connection with other molecules, which stabilizes bacteriocin activity. Bacteriocin action was steady at 37°C with pH of 2.0 to 10.0 after incubation (P0.05). This shows that bacteriocin be able to treat both acidic and non-acidic foods. Bacteriocin's antimicrobial efficacy was not inactivated at high temperatures, such as boiling (60°C, 30 minutes) or boiling (100°C, 45 minutes).

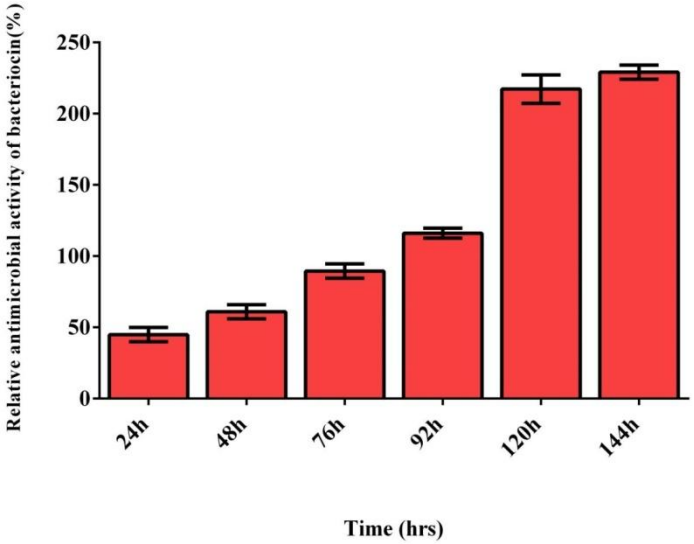

Figure 2 C- Stability of bacteriocins with time interval

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Molecular weight determination of bacteriocin: The bacteriocin's molecular weight was measured by Tricine-SDS PAGE (Fig. 4.8). The protein band was visible when dyed with Coomassie blue, and bacteriocin's antimicrobial activity was linked to a band with the respective molecular weight of around 14.5kDa.

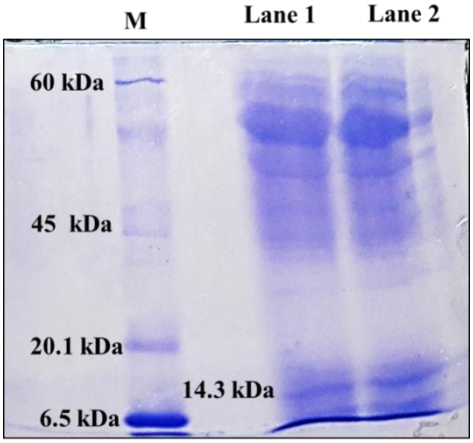

Figure 3 - Tricine SDS-PAGE analysis of bacteriocin: Lane1 indicates the marker and lane 2, 3 indicates bacteriocin.

Amino acid sequence of bacteriocin: The N terminus of distilled bacteriocin begins with valine and ends with lysine, according to the amino acid series.This band's amino acid analysis yielded the following partial sequence: "VYAEPQAQNIPWVK." The molecular mass was found to be 14300 Da, with a pH of 8.77 as the isoelectric point. The bacteriocin had four hydrophobic residues and a total charge of two. The Bactibase Database was used to determine the theoretical isoelectric point 5.97 (pI), molecular weight, amino acid composition, grand average of hydropathicity of the peptide chain (GRAVY)and atomic composition.

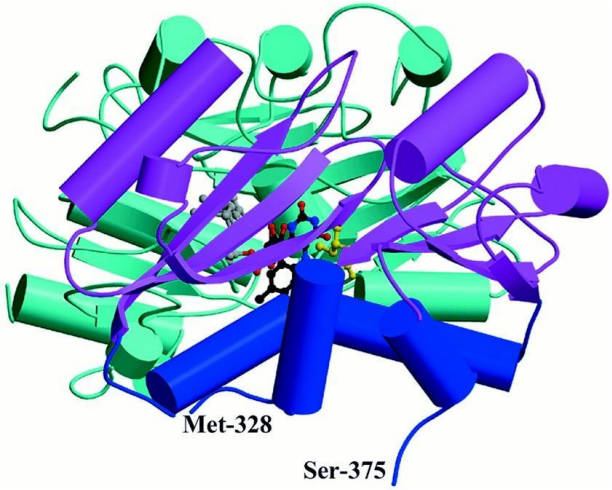

Figure 4 –The structure prediction of bacteriocin from *Lactobacillus delbrueckii*

Bacteriocin Identification by MALDI/ToF: Membranes to adsorb bacteriocins were successfully analyzed using MALDI-TOF MS. The on-target washing process, on the other hand, has been shown to be auseful and efficient technique for the revealing of bacteriocins by MALDI-TOF MS.Figure4 displays the spectrum of supernatant taken from the culture of*Lactobacillusdelbrueckii*. Some pollutants were removed with a ten-second wash with milli-Q

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water; however, a 30-second wash was found to be the most effective, resulting in a peptide signal with greater power, improved resolution, and less noise.The 60-second wash eliminated the pollutants as well, but it seemed to weaken the sample signal. Despite the fact that some of the samples had low signal strength and resolution.With signals ranging from 3,350 to 3,360 Da, the m/z ratios of all the samples were close to the mass predicted for nisin (MH+ = 3,354 0.1 percent). The single band on native-PAGE illustrated the homogeneity of the distilled bacteriocin.Gel filtration was used to assess the molecular mass of bacteriocin, which was found to be 14.3KDa. The monomer bacteriocin has a molecular mass of 14.3 kD, as calculated by MALDITOF-MS fingerprinting, SDS-PAGE, and gel filtration analysis.The molecular mass of bacteriocin was found to be close to that of other bacteriocins reported from Bacilli, such as Colicins, a large bacteriocin with a various molecular weight (25-80kDa) that has also been identified as an endopeptidase that exerts its inhibitory action by damaging the cell wall of sensitive bacteria, thus being classified as a cell wall degrading bacteriocin.Using the MASCOT and PROWL servers, the observed mass and relative intensities obtained from peptide mass fingerprinting were used to classify the parent sequence against profiles of recognized sequences (Figure 5.3-5.4). The isolated bacteriocin was found to be identical to Lactobacillus delbrueckii uracil-5-methyltransferase (MASCOT search score: 68) with a molecular weight of 50kDa.A number of high molecular weight bacteriocins that target sensitive bacteria's cell walls were also well defined. These bacteriocins, also known as endopeptidase or bacteriocins, were graded as class IIIabacteriocins (Clara et al., 2012). FemABX-like proteins function as peptidyltransfereases, allowing amino acids to be incorporated into the peptidoglycan's interchange peptide connection, resulting in the targeted bacterial indicator's cell wall lysis. The amino acid sequence of bacteriocin was compared to the protein sequence of *Lactobacillus delbrueckii* uracil-5-methyltransferase using MALDI-TOF-MS. The incomplete N terminal sequence of bacteriocin (the first 14 amino acid residues) is VYAEPQAQNIPWVK.

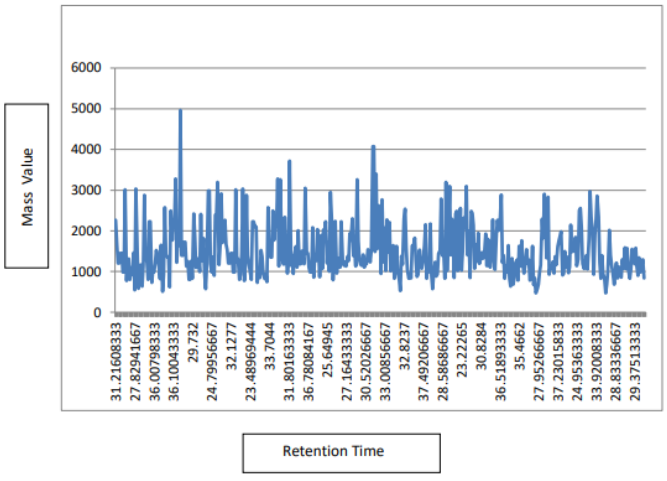

Figure – 5 MALDI-TOF spectrum of bacteriocin

Bio assays of bacteriocin

Antifungal efficacy: Thus, the isolate *Lactobacillus delbrueckii's* wide spectrum antimicrobial effect against common pathogenic bacteria suggested that this isolate could be useful and could be used to isolate and purify bacteriocin or bacteriocin-like inhibitory molecules.The isolated

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strain and eventually purified bacteriocin showed significant reticent effects on Candida albicans in the current research. Highest antifungal efficacy was observed in *Lactobacillus delbrueckii*. This showed that bacteriocin derived from *Lactobacillus delbrueckii* had a wide spectrum of action against Candida albicans.*Lactobacillusdelbrueckii* metabolites had inhibitory zones ranging from 16 to 22 mm.Inhibitory zones of 18 and 17 mm, respectively were recorded in *Aspergillusniger* culture when indicted against bacteriocin but only 12 mm zone was observed against the same fungus with fluconazole anti-fungal after the incubation of 72 h.

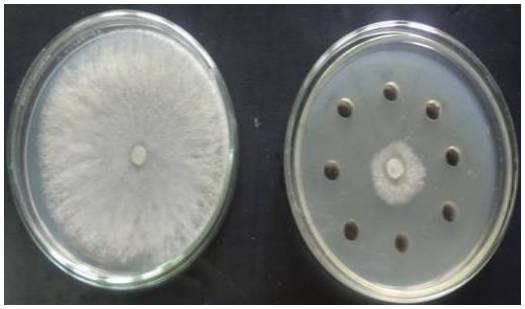

Figure -6 Antifungal activity – Growth inhibition

Anti-cancer potential of bacteriocinpeptides: Five various human cancer cells, including MCF-7, HeLa,HT1080, HEK293T andH1299 were used to assess the anticancer efficacy of bacteriocin LACDEL-14.3.The cell lines were incubated with higher concentrations of the bacteriocin LACDEL-14.3 in the specific growth medium which showsthat LACDEL-14.3 has a natural cytotoxic effect against whole cancer cell lines studied. Whole cancer cell lines displayed cytotoxicity which depends on the bacteriocinconcentration.The highest activity was observed against MCF-7 cells at a concentration of 10 μM. At a 10 μM concentration LS10, MCF-7 showed 60% cytotoxicity, while HT1080, HEK293T, and H1299exhibited just 40% cytotoxicity. However, at a concentration of 10 μM, there was no significant cytotoxicity in HeLa cells, but at a concentration of 100 μM, there was 50% cytotoxicity in HeLa and other cell lines.

Figure – 7 Anti-cancer activity of bacteriocin against HUH -7 cell lines

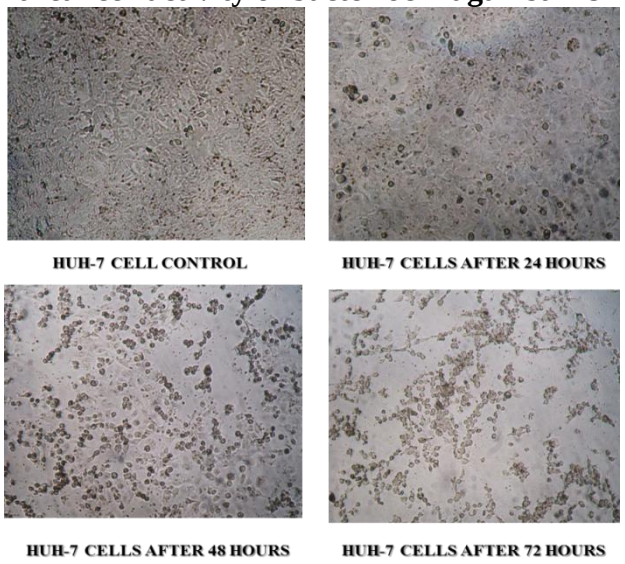

Figure – 8 Anti-cancer activity of bacteriocin against HUH -7 cell lines morphology

Conclusion: These findings help *Lactobacillus delbrueckii's* probiotic efficacy in vaginal application and further pathologies. In fact, our current study and findings are the first to identify a new bacteriocin which was produced by *Lactobacillus delbrueckii*which molecular weight was predicted as 13 kDa and antimicrobial efficacy against vaginal pathogenic microorganisms, specifically Candida and the most common pathogens associated with vaginal infections. These findings support the use of this probiotic strain in clinical practice for the treatment and prevention of infections by establishing a novel mechanism of action.More research into bacteriocin synthesis, isolation, and purification, as well as its possible mechanism of action, is needed.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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