Antimicrobial Potential of Bacteriocin from *Lactococcus lactis* RGUSCJ08 Against Uropathogens

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KEYWORDS
Bacteriocins, Probiota, Uropathogens, Lactococcus Lactis, Antimicrobial Activity

ABSTRACT
Introduction: Urinary tract infections caused by uropathogens pose a significant global health challenge, often leading to antimicrobial resistance. Bacteriocins from lactic acid bacteria are emerging as promising alternatives to traditional antibiotics. This study isolates and evaluates a bacteriocin-producing Lactococcus lactis strain for its antimicrobial activity against uropathogens.
Materials and Methods: Samples from women (18–24 years) were tested via the spot-on-lawn method, where supernatants were spotted against E.coli, Staphylococcus aureus, Streptococcus pyogenes, Enterococcus faecalis, and Klebsiella pneumonia. One sample showed inhibitory zones, suggesting the presence of a potential probiotic strain. The strain Lactococcus lactis RGUSCJ08 was identified using 16S rDNA sequencing, and used for bacteriocin production. The bacteriocin was purified through ammonium sulfate precipitation, dialysis, and FPLC. Using a spot assay to evaluate their antibacterial activity, purified bacteriocin samples were tested against uropathogens (Escherichia coli, Streptococcus pyogenes, Enterococcus faecalis, and Klebsiella pneumoniae). SEM analysis was performed to observe and highlight the bacteriocin's mode of action against uropathogens.
Results: RGUSCJ08 exhibited inhibitory activity against the tested pathogens except Escherichia coli. Scanning electron microscopy (SEM) revealed structural damage to the target pathogens, supporting its antimicrobial efficacy.
Conclusion: The bacteriocin from Lactococcus lactis RGUSCJ08 shows significant potential as a natural antimicrobial agent against uropathogens. Its effectiveness and stability highlight its application in developing alternative therapies for multidrug-resistant infections. Further studies on its detailed mechanism of action and therapeutic applicability are warranted.

1. Introduction:

Urinary tract infections (UTIs) that are caused by uropathogens pose a significant global health challenge and often contribute to antimicrobial resistance. The significance of maintaining a healthy probiotic microbiota within the female reproductive tract is well-documented give that it plays an essential role in the prevention of infections and the maintenance of general health [1]. The probiotic microorganisms, particularly lactic acid bacteria, often produce bacteriocins known to be antimicrobial peptides that often inhibit the growth of the pathogenic bacteria.

Bacteriocins are routinely defined as ribosomally synthesized antimicrobial peptides that are produced by bacteria and can kill and in some cases inhibit the growth of other bacteria. The peptides are effective given that they offer potential solutions to the rising problem of uropathogens and the rising problem of antibiotic resistance [2], [3]. Over the years traditional antibiotics have been the mainstay in the treatment of UTIs. Nonetheless, their overuse and misuse have contributed to the emergence of multidrug-resistance strains and this necessitates the search for alternative treatments that are not only effective but sustainable.

The bacteriocins from lactic acid bacteria have emerged as promising alternatives because of their specificity and low propensity for the development of resistance. The assertion is due to the fact that naturally occurring antimicrobial agents can often be harnessed to combat uropathogens without any adverse effects that are associated with conventional antibiotics [4], [5]. The potential of bacteriocins to be integrated into existing healthcare practices makes them an attractive option in the development of new therapeutic strategies.

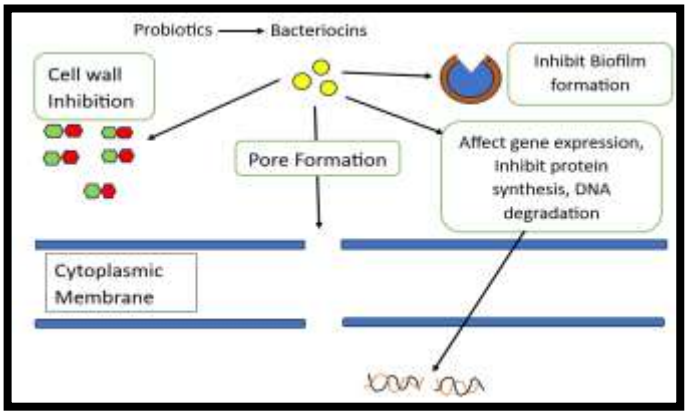

Figure 1: Bacteriocins activity against uropathogens

The primary objective of the study is to isolate and evaluate a bacteriocin-producing lactic acid bacteria (LAB) strain for its antimicrobial activity against uropathogens. The study seeks to identify a viable alternative to traditional antibiotics that can be incorporated into primary healthcare settings and in the long term reduce the prevalence of UTIs while improving patient outcomes. The findings from this study are likely to pave the way for new and effective treatments for multidrug-resistant infections and thus contribute to the global fight against antibiotic resistance.

2. Objectives

- To isolate and identify a lactic acid-producing bacterial strain capable of producing bacteriocins with inhibitory effects against common uropathogens.
- To examine the bacteriocin's antimicrobial properties purified from the isolated strain.
- To examine the structural changes induced by the bacteriocin on target pathogens using scanning electron microscopy (SEM), thereby gaining insights into its mode of action.

3. Materials And Methods

Sample Collection and Ethical Considerations

The study was conducted with 115 female participants within the reproductive age range of 18 to 40 years. Participants who were menstruating or undergoing antibiotic treatment at the time of the study were excluded. Vaginal samples were collected using sterile Pasteur pipettes, following the acquisition of informed consent from each participant. The procedure was carried out only after ensuring that the participants were fully comfortable and with prior ethical approval from the Ethical Committee of the government medical hospital. The collected swabs were immediately transferred to 2 ml Eppendorf tubes containing sterile water for storage. The stored sample was taken to the lab for test within 2 hrs.

Sample analysis

The collected samples were centrifuged to obtain the supernatant, which was subsequently tested for antimicrobial activity using the spot-on-lawn method. The assay was performed against uropathogenic indicator strains, including Escherichia coli (MTCC 77), Klebsiella pneumoniae (MTCC 39), Staphylococcus aureus (MTCC 87), Enterococcus faecalis (MTCC 3159), and Streptococcus pyogenes (MTCC 1925). Nutrient agar was used as the base medium for the assay. A 1% soft agar suspension containing the indicator strain was evenly layered over a 2% nutrient agar plate to create a bacterial lawn. The supernatant of the sample was carefully spotted onto the surface of the prepared plates. The plates were incubated overnight at 37ºC, and the formation of inhibitory zones around the spots was observed to determine antimicrobial activity [2][3].

The inhibition index was calculated by applying the following equation-

$IP = (\Phi ClearZone - \Phi PlugSize)/\Phi PlugSizeIP$

where IP is the inhibition index, and Φ is the diameter (mm).

Selection of samples containing potential probiota

Samples exhibiting a clear zone of inhibition against uropathogenic indicator strains were selected for further analysis. The selected samples were serially diluted in distilled water to reduce microbial density. Aliquots from appropriate dilution levels were plated onto MRS agar plates, a selective medium for lactic acid bacteria [1]. The plates were incubated at 37°C for 24–48 hours under anaerobic conditions to promote the growth of lactic acid bacteria. Distinct colonies from the MRS agar plates were sub-cultured using the streak plate method to obtain pure isolates. Isolates were re-tested for antimicrobial activity against the uropathogenic indicator strains (Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Enterococcus faecalis, and Streptococcus pyogenes) using the spot-on-lawn method. Optical density (OD) at 600 nm was measured at regular intervals during the growth of the isolates in MRS broth to assess their growth kinetics.

Identification:

A. **Gram Staining**: Isolated colonies were Gram-stained to confirm the presence of Gram-positive, rod-shaped bacteria were observed over a phase contrast microscope.

B. **Colony Morphology**: Colonies on MRS agar were identified based on their smooth, convex, and translucent appearance.

C. **Catalase Test**: Isolates were subjected to the catalase test by adding a drop of 3% hydrogen peroxide to a bacterial smear. The absence of bubble formation confirmed catalase-negative bacteria, characteristic of Lactobacillus [4]

D. **pH and Amine Test**: A saline suspension of the sample was applied to a glass slide, followed by the addition of one drop of KOH. The slide was gently fanned to detect volatile amines, characterized by a fishy odor. The pH of the sample was measured using a pH meter [4].

E. **H_2O_2 Production**: Isolates were tested for H_2O_2 production by growing them anaerobically on agar containing horseradish peroxidase and TMB. Blue pigmentation indicated H_2O_2 production due to the oxidation of TMB [4].

F. **Bile Salt Tolerance**: Isolates were cultured in MRS broth containing bile salts at concentrations of 0.3%, 0.5%, and 1%. Growth was observed after 24–48 hours of incubation at 37°C to assess bile salt tolerance [4].

G. **CO_2 Production**: Homofermentative isolates were characterized for CO_2 production from glucose. MRS broth with inverted Durham tubes was inoculated with 50 µl of overnight culture and incubated at 37°C for five days. Gas formation in the Durham tube confirmed CO_2 production [4].

H. **NaCl Tolerance**: Isolates were cultured in MRS broth with NaCl concentrations of 2%, 4%, and 8%. Growth was monitored after incubation at 37°C [4].

I. **Antimicrobial Activity**: The isolate's antibacterial activity was tested using the agar spot-on-lawn method against the indicators. Plates were incubated aerobically at 37°C for 24 hours, and inhibition zones were measured. Isolates producing significant halos were selected for further analysis [2].

J. **Selection of Potent Strains**: Isolates exhibiting antimicrobial activity and demonstrating desirable traits such as bile salt and NaCl tolerance were selected for 16S rDNA sequencing to confirm their identity [4].

Bacteriocin Purification:

Production of Crude Bacteriocin:

- Inoculation and Incubation: 300 ml of MRS broth was inoculated with 1% v/v overnight culture of

Lactobacillus isolates and incubated anaerobically at 37°C for 48 hours.

- Centrifugation: Cells were separated by centrifugation at 10,000 rpm for 10 minutes.
- pH Adjustment: The supernatant was adjusted to pH 6.5 using 1N NaOH to neutralize organic acids.
- H_2O_2 Elimination: Catalase (5 mg/ml) was added to eliminate inhibitory effects of hydrogen peroxide.
- Filtration: The cell-free supernatant was filtered through a 0.45 µm membrane to obtain crude bacteriocin [6].

Ammonium Sulfate Precipitation:

- 90% saturation was achieved by dissolving 90.45 g ammonium sulfate in the cell-free supernatant with gentle stirring.
- The precipitate was collected by centrifugation and redissolved in 6 ml of 0.05 M sodium phosphate buffer (pH 7.0).
- The solution was stored under cold conditions for further use [5], [6].

Dialysis:

- The bacteriocin solution was transferred to dialysis bags and tightly sealed.
- Dialysis was performed in 0.05 M sodium phosphate buffer (pH 7.0) for 18 hours at 4°C, with buffer changes every 6 hours.
- The dialyzed solution was adjusted to pH 6.5 using NaOH and sterilized through a 0.45 µm membrane filter.

SDS-PAGE Analysis:

- Preparation: 25 µl of the bacteriocin sample was mixed with 25 µl loading buffer (100 mM Tris base, 100 mM glycine, 4% SDS, 8 M urea, 0.01% bromophenol blue) and heated at 100°C for 3 minutes.
- Electrophoresis: The sample was run on a 16% SDS-polyacrylamide gel at 35 mA for the stacking gel and 50 mA for the resolving gel. A protein marker (2–45 kDa) was included.
- Staining: Gels were stained with Coomassie brilliant blue to visualize protein bands.

Antibacterial Activity Detection on Gel:

- The gel was washed three times in 0.1% Tween 80 (40 minutes each).
- Soft nutrient agar containing indicator strain cells was overlaid on the gel.
- Clear zones indicating antibacterial activity were observed after incubation at 30°C overnight [6], [7].

Protease sensitivity assay:

To assess the sensitivity of bacteriocin to proteolytic enzymes, 200 µl of bacteriocin solution was separately treated with 1 mg/ml of proteinase K, and α-chymotrypsin. The mixtures were incubated at 37°C for 2 hours, and the enzyme-treated samples were tested for antimicrobial activity using the spot-on-lawn method against indicator strains. Untreated bacteriocin served as a control to evaluate activity loss, confirming the proteinaceous nature of the bacteriocin [7].

Heat stability- The bacteriocin was introduced to various temperatures from 50^{o}C, 70^{o}C, 100^{o}C, 120^{o}C, and 150^{o}C to check its tolerance to high temperatures.

Scanning Electron Microscopy to observe mode of action:

For SEM analysis, Lactococcus lactis RGUSCJ08 (the laboratory isolated strain), E. faecalis, S. aureus, S. pyogenes, and K. pneumoniae were subcultured in 5 ml nutrient broth across nine tubes (4 controls, 4 treated with bacteriocin, and 1 L. lactis RGUSCJ08). Subcultures were treated with bacteriocin at a

5 µl/ml concentration and incubated for 24 hours. The cultures were then centrifuged to collect bacterial pellets, which were fixed with 2.5% glutaraldehyde overnight at 4°C. Fixed samples were dehydrated through sequential ethanol washes (20%, 40%, 60%, 80%, and 100%), mounted on SEM stubs, and visualized under SEM at varying magnifications to observe the bacteriocin's mode of action [8]. E.coli was not taken for SEM as the bacteriocin didn't show any zone of inhibition against it.

4. Results

A Gram-positive strain with antimicrobial potential against uropathogens was identified. Following extensive screening and 16S rDNA sequencing, the strain was confirmed as a lactic acid-producing Lactococcus lactis, designated RGUSCJ08. The key identification characteristics of the strain are summarized in the table below:

Table 1. Key characteristics of the strain RGUSCJ08

Characteristic	Result
Gram Staining	Positive (+)
Catalase Test	Negative (-)
Bile Salt Tolerance	Positive (+)
CO_2 Production	Positive (+)
Homofermentative Nature	Positive (+)
H_2O_2 Production	Positive (+)
NaCl Tolerance (6.5%)	Positive (+)
Antimicrobial Activity	Positive (+)
pH Test	Normal vaginal pH (4.0–4.5)
Amine Test	Negative (No fishy odor)

Photographs under Phase Contrast Microscope (100 x)

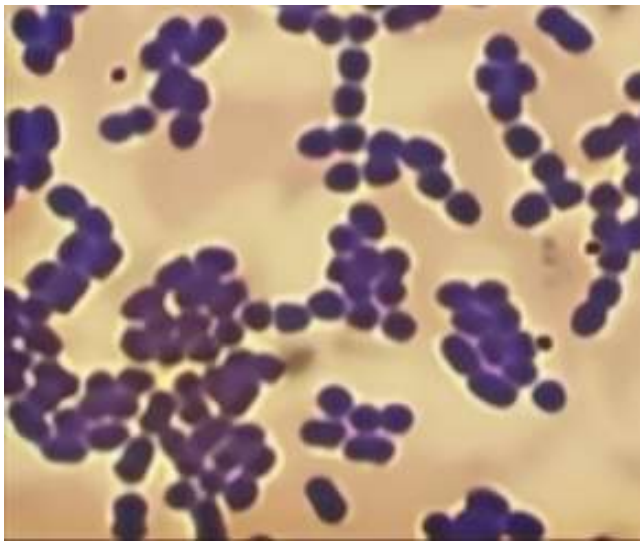

Figure 2: Lactococcus lactis RGUSCJ08 strain

Figure 3: Sample under microscope

Phylogenetic tree

The 16S rDNA sequencing result shows a close resemblance to Lactococcus lactis (100% match). A phylogenetic tree was constructed using MEGA software to determine the evolutionary relationship of the isolated strain Lactococcus lactis RGUSCJ08 with other closely related species [9]. The analysis revealed that the strain clustered closely with reference strains of Lactococcus lactis, confirming its taxonomic identity. The high bootstrap values in the phylogenetic tree supported the reliability of the evolutionary relationships depicted. This analysis further validated the identification of RGUSCJ08 as a distinct strain of Lactococcus lactis**.**

Figure 4: Phylogenetic tree

Antimicrobial activity tests.

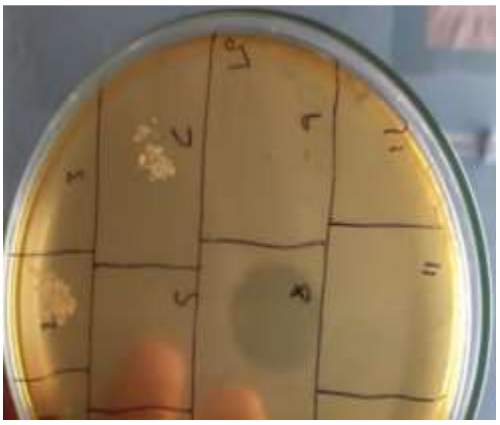

Figure 5: Antimicrobial activity of sample supernatant on uropathogenic indicators

Figure 6: S. aureus

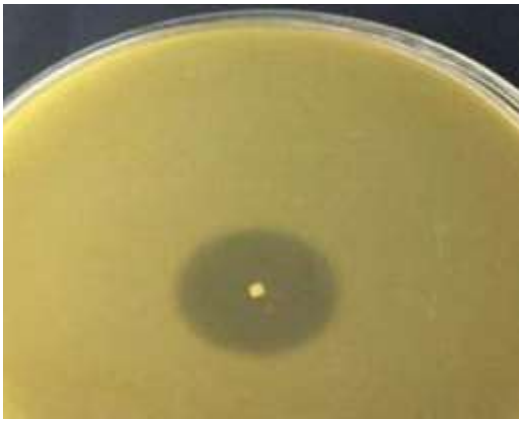

Figure 7: E. faecalis

Figure 8: S. pyogenes

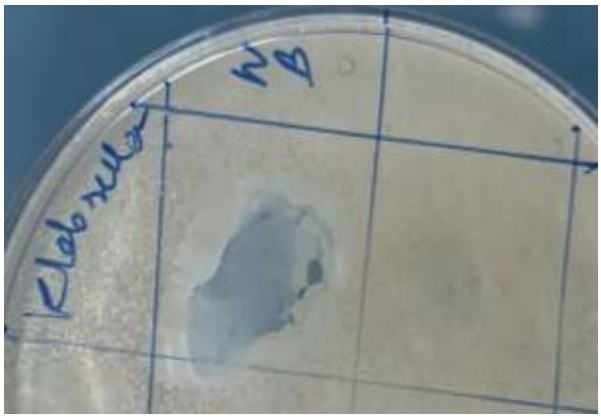

Figure 9: K. pneumonia

Figure 10: E.coli

Table 2: Zone of inhibition results for Lactococcus lactis RGUSCJ08

Pathogen	Zone of Inhibition (mm)	Result
Escherichia coli (MTCC 77)	0 mm	Negative (-)
Klebsiella pneumoniae (MTCC 39)	12 mm	Positive (+)
Staphylococcus aureus (MTCC 87)	18 mm	Positive (+)
Enterococcus faecalis (MTCC 3159)	13 mm	Positive (+)
Streptococcus pyogenes (MTCC 1925)	14 mm	Positive (+)

Lactococcus lactis RGUSCJ08 demonstrated effective antimicrobial activity against four Gram-positive pathogens, with the largest zone (18 mm) observed for Staphylococcus aureus. No inhibitory activity was observed against Escherichia coli.

Proteinaceous nature of the antimicrobial compound- Loss of activity after treatment with proteolytic enzymes shows the proteinaceous nature of the antimicrobial compound. This antimicrobial compound is resistant to high temperatures. It was able to sustain its antimicrobial activity till 120^{o}C.

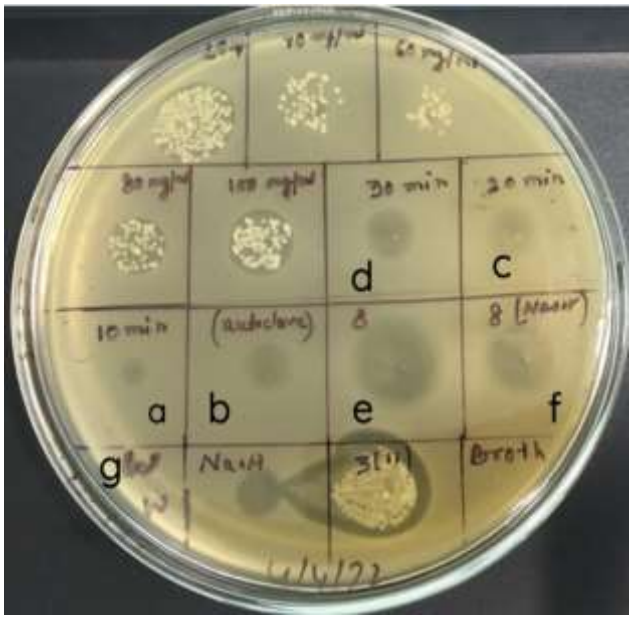

Figure 11: Heat stability nature of the antimicrobial compound. (a) Boiling water bath for 10 minutes; (b) Bacteriocin activity after autoclaving at 121.5ºC; (c) Activity after 20 minutes boiling; (d) Activity after 30 minutes of boiling in a water bath; (e) Bacteriocin activity at 37ºC; (f) NaOH (g) Broth without bacteriocin

Growth curve and bacteriocin activity: Spot & lawn at different time intervals using the pathogenic indicators on the Nutrient agar plate was done. The highest inhibitory zone is observed in 8–10-hour growth i.e., during the late exponential stage to early stationary phase. After which zone is constant (studied up to 24hr).

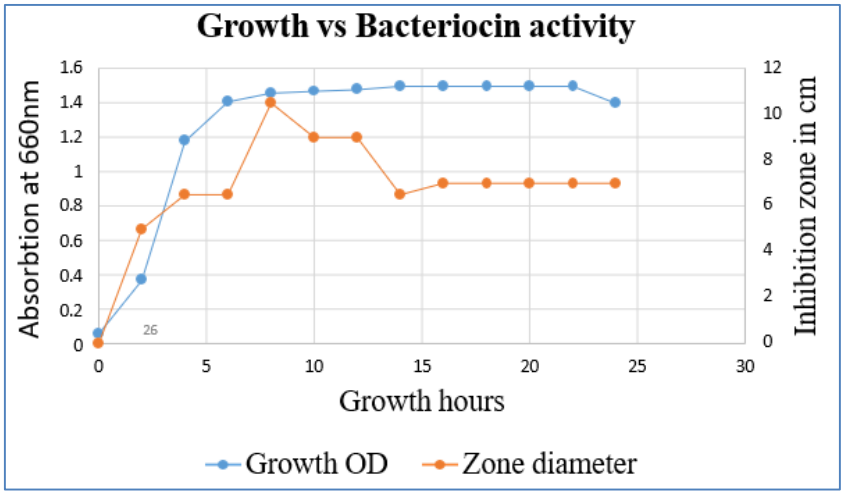

Figure 12: Growth vs. Bacteriocin activity

FPLC purification: Prominent peak was observed at 119.12 ml, corresponding to the elution of the active bacteriocin fraction. This purified fraction collected was responsible for the antimicrobial effects observed in assays against the uropathogens [10].

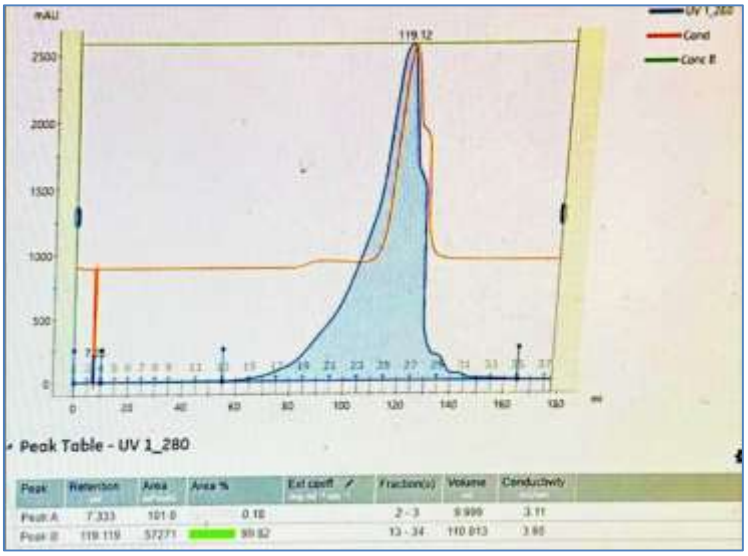

Figure 13: FPLC purification

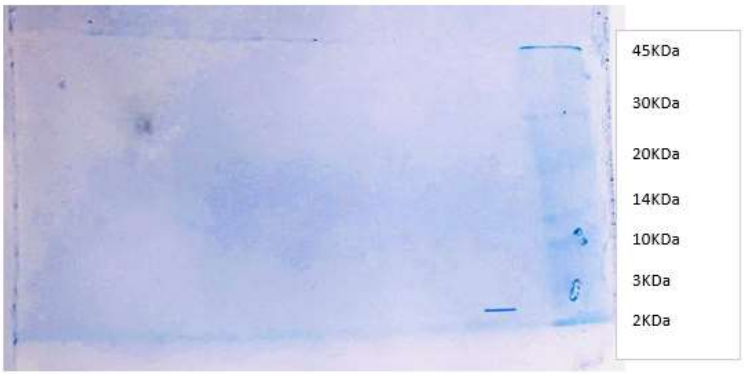

Figure 14: The bacteriocin purified gave bands at 2.5 kDa

Antimicrobial activity against different pathogenic indicators.: The Arbitrary Units (AU) were determined by spot on lawn method using the uropathogens as indicator strains. The zone of inhibition was observed [9]. The AU was defined as the reciprocal highest dilution showing a clear zone of growth inhibition. The bacteriocin-like protein activity was determined as AU/ml (AU/ml was defined as the reciprocal of the highest bacteriocin-like protein dilution factor that inhibited the growth of the indicator strain). The activity was calculated by using the formula below. AU/ml = (X × 1,000)/V; X is the highest dilution exhibiting inhibition V is the volume of filled sample bacteriocin. Dilutions from ½ to 1/150 were observed [9]. The following are the results for each pathogen-

Table 3: Antimicrobial activity (AU/mL) of the bacteriocin against the pathogens

Indicator	Specific activity (AU/mL)
Staphylococcus aureus	2000
Enterococcus faecalis	1300
Streptococcus pyogenes	1700
Klebsiella pneumonae	1100
Escherichia coli	0

Table 4: Activity (AU/mL) of the bacteriocin against pathogens decreased after the chymotrypsin and protease K treatment.

Indicator	Specific activity (AU/mL) after chymotrypsin treatment	Specific activity (AU/mL) after Protease K treatment
Staphylococcus aureus	0	0
Enterococcus faecalis	0	10
Streptococcus pyogenes	0	0
Klebsiella pneumonae	0	0

SEM images: Images were observed in 24500 X magnification and 1µm.

Formed pore in cell wall of E. faecalis cells treated with the purified bacteriocin.

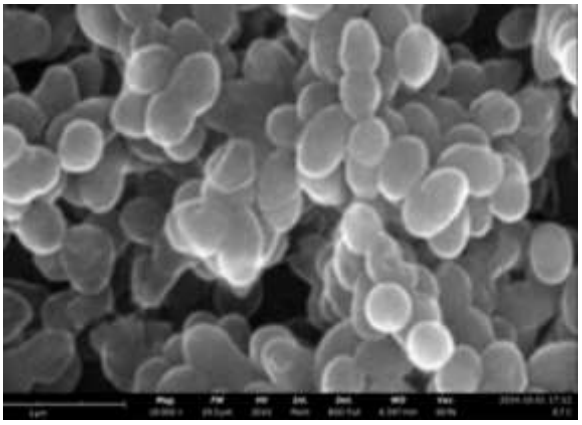

Figure 15 a: E.faecalis control.

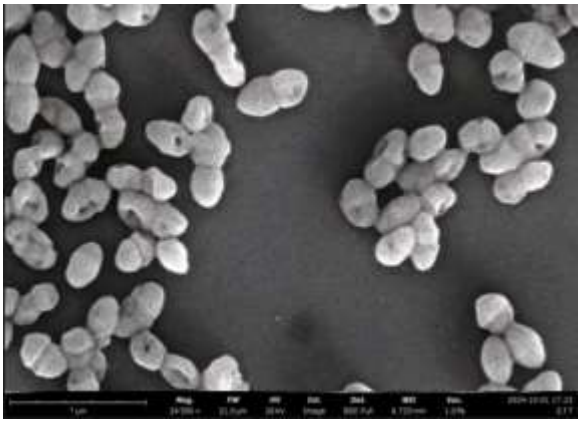

Figure 15 b: E.faecalis treated.

K.pneumonea cell wall disruption, cell wall breakdown in treated cells could be observed in SEM.

Figure 16 a: K.pneumonea control

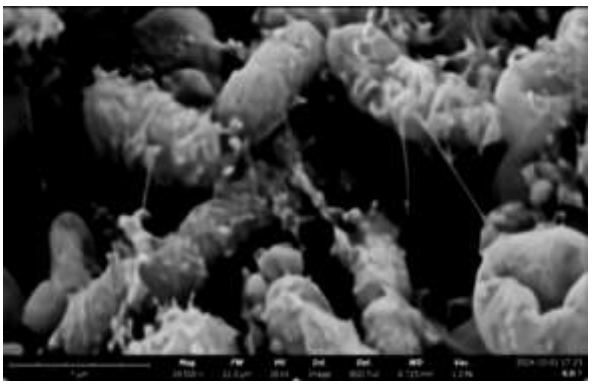

Figure 16 b: K.pneumonea treated

Cell wall disruption can be observed in the treated cells of S.pyogenes when compared with the untreated ones.

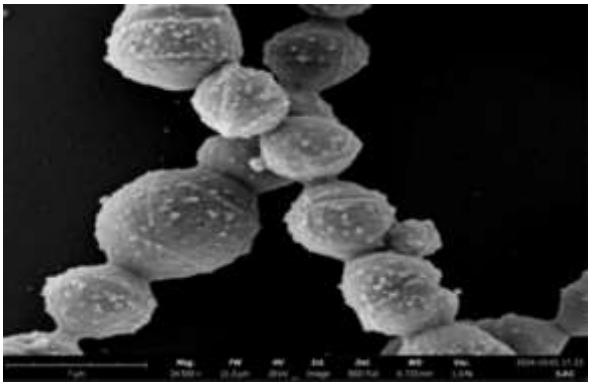

Figure 17 a : S.pyrogens control

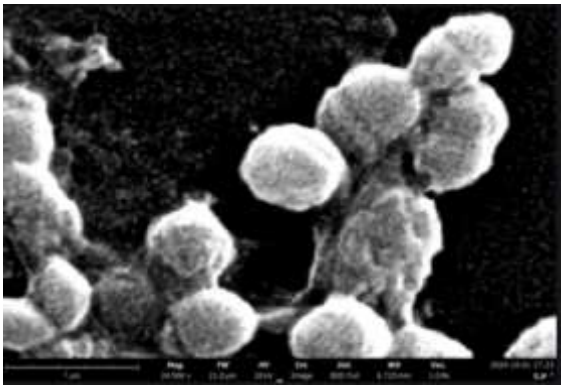

Figure 17 b: S.pyrogens treated.

Disruption of cell wall and cell structure of treated cells of *S. aureus* can be noted.

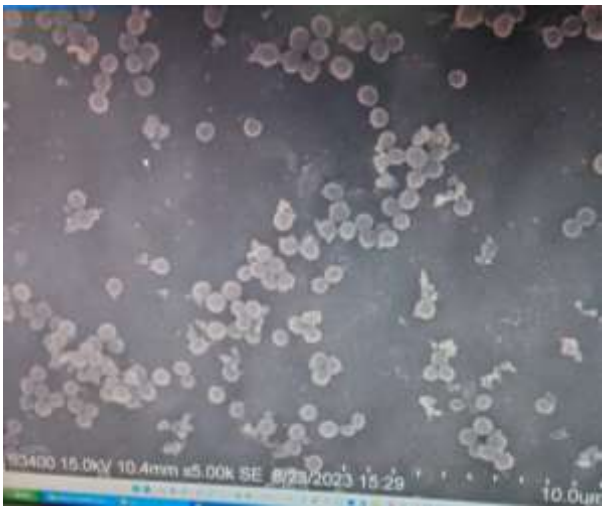

Figure 18 a: S.aureus control treated.

Figure 18 b: S.aureus treated.

5. Conclusion

The study successfully identified Lactococcus lactis RGUSCJ08, a Gram-positive, lactic acid-producing bacterium isolated from vaginal samples. The strain exhibited potent antimicrobial activity against Gram-positive uropathogens, including Klebsiella pneumoniae, Staphylococcus aureus, Enterococcus faecalis, and Streptococcus pyogenes, but showed no effect on Escherichia coli.

The bacteriocin produced by RGUSCJ08 was purified and partially characterized, with a molecular weight of approximately 2.5 kDa, confirmed by SDS-PAGE. Functional analysis, including proteolytic enzyme treatments, demonstrated its proteinaceous nature [11][12]. SEM analysis revealed pore formation in E. faecalis and cell wall disruption in other Gram-positive pathogens, further elucidating the mode of action of the bacteriocin [13]. Cell wall disruption was also observed in Gram-negative K. pneumonea [14].

In conclusion, bacteriocins produced by lactic acid-producing bacteria offer promising alternatives to conventional antibiotics, fostering new strategies against antimicrobial resistance. Additionally, their combined use with traditional antibiotics may further enhance their overall therapeutic potential as reported in various studies [15]. The results in our study suggest that Lactococcus lactis RGUSCJ08 and its bacteriocin hold potential for development as a biotherapeutic agent against uropathogens, particularly in addressing infections caused by Gram-positive and Gram-negative bacteria. Further studies are warranted to explore its therapeutic applications against E.coli, a common uropathogen, by the addition of different metabolites or enzymes to enhance its activity [16] and to identify the precise molecular mechanisms of its antimicrobial action.

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