

Evaluation of bio-preservative potential of bacteriocins extracted from *Lactobacillus spp*

Abstract

Bacteriocins produced by Lactic Acid Bacteria (LAB) have garnered significant attention for their potential as natural and safe preservatives in the food industries, being Generally Regarded as Safe (GRAS) and typically non-toxic. This study aimed to isolate, partially purify, and characterize bacteriocins from LAB isolated from milk and yogurt samples. Initial screening of bacteriocin producing microbes was performed by agar-overlay method against *Staphylococcus sp.* Bacteriocin activity assay was performed using agar-well diffusion method against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella typhi* by determining their zone of inhibition. The proteinaceous compound was isolated from cell-free culture supernatant and was partially purified by 60% ammonium sulphate precipitation and was further purified by extraction with chloroform-methanol (2:1 v/v), followed by molecular identification using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS PAGE) and characterization of its stability under various conditions. Finally, the bacteriocin preparation was sprayed on beef sample and was evaluated up to 48 hours. Two of the isolates demonstrated significant antimicrobial activity against all four indicator bacteria. The bacteriocins were stable across various pH levels, temperatures, and proteolytic enzyme treatments. After 12 h, it exhibited significant microbial inhibition (Control: TBC 2.55×10^8 CFU/g and CC 1.92×10^6 CFU/g; Sample: TBC 2.2×10^8 CFU/ml and CC 1.8×10^6 CFU/ml). Thus, the isolated LAB strains exhibit potential for bacteriocin production, offering promising applications in food preservation and safety, particularly on meat and meat products.

Keywords: bacteriocins, lactic acid bacteria, antimicrobial activity, food preservation, sds page, ammonium sulphate precipitation, agar-well diffusion method.

Volume 18 Issue 1 - 2025

Anika Maharin Roza, Nazmul Rahman Chowdhury, Narayan Chandro Sarker Nirob, Kazi Mohammed Didarul Islam, Md. Emdadul Islam

Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna-9208, Bangladesh

Correspondence: Md. Emdadul Islam, Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna-9208, Bangladesh, Tel +880-1712-773266, Email emdad@bge.ku.ac.bd

Received: December 27, 2024 | **Published:** January 14, 2025

Introduction

Bacteriocins are proteinaceous toxins produced by bacteria.¹ Their primary function is to inhibit the growth of similar or closely related bacterial strains. Bacteriocins made by LAB have gained a lot of attention due to their potential use as natural and safe preservatives in food and pharmaceutical industries. The bacteriocins produced by LAB are “Generally Regarded as Safe” (GRAS)² and have gained significant interest as a new method for controlling infections in food products. LAB have long been utilized as starter cultures in the fermentation process of various foods and beverages, owing to their ability to enhance flavor, aroma, and retard spoilage.³ The preservative effect of LAB is primarily attributed to the acidic environment they create during their growth phase. However, their preservative capabilities extend beyond the production of lactic and acetic acid. LAB can produce and secrete a variety of inhibitory substances, including hydrogen peroxide, ethanol, diacetyl, carbon dioxide, substances akin to bacteriocins or antibiotics, and bacteriocins themselves.⁴

The increased consumption of foods containing additives formulated with chemical preservatives and consumer concerns have created a higher demand for more natural and minimally processed foods, therefore, there is a high interest in naturally produced antimicrobial agents that do not produce adverse effects.⁵ In this study we tried to isolate, identify and characterize bacteriocin isolated from milk and yogurt sample and evaluate the bio-preservative potential of partially purified bacteriocin using various biochemical and molecular techniques.

Materials and methods

Sample preparation

Milk and yogurt samples from local sweet shops in Gollamari, Khulna, Bangladesh were collected, diluted (10^{-1} - 10^{-6}), and plated (0.1 ml suspension) on MRS agar plates.⁶ After incubation at 37°C for 48-72h, single colonies were sub-cultured until pure. A culture stock was prepared with a 50% glycerol solution, mixed with the bacterial culture, and stored at -20°C.

Initial screening for antagonistic activity

All LAB cultures isolated from MRS were submitted to an initial screening to verify the presence of antagonist activity using the Agar overlay method.⁷ In brief, LAB isolates were grown overnight and then stabbed onto MRS agar plates (1.5% agar), followed by incubation at 37°C for 3 hours. Subsequently, soft agar (0.8% agar) containing the indicator strain at a concentration of 10^6 CFU/mL was layered over the plates, which were then incubated at 37°C for 24 hours. The appearance of clear inhibition zones around the colonies signified antimicrobial activity.

Identification of bacterial isolates

Physiological and morphological tests were conducted following standard methods to identify bacterial isolates, encompassing characteristics such as size, shape, color, and texture. Microscopic examination was done by Gram staining. Biochemical tests included the catalase test, oxidase test, citrate utilization, TSI test and urease test. Additionally, production of bacteriocin was examined by

propagating isolated strains in MRS broth, followed by centrifugation to obtain cell-free supernatant, adjusted to pH 6.5, and treated to prevent proteolytic degradation, serving as crude bacteriocin.

Production of bacteriocin

The isolated strain was propagated in MRS broth seeded with 1% (v/v) inoculum of overnight culture and maintained at optimized culture condition for 48 h at 37°C. After incubation, cells were removed from the growth medium by centrifugation at 6000 rpm for 20 minutes, 4°C. The cell-free supernatant (CFS) was adjusted to pH 6.5 using 1 mol/L NaOH and it was used as crude bacteriocin. To avoid proteolytic degradation of the bacteriocin, CFS was treated for 10 min at 80°C.⁸

Antimicrobial activity assay

The bacteriocin’s antimicrobial activity was tested against *E. coli*, *S. aureus*, *S. typhi*, and *P. aeruginosa* using the agar well diffusion method.⁹ The indicator bacteria were cultured on Nutrient Agar, inoculated into Nutrient Broth, and incubated. Muller-Hinton agar plates were prepared and inoculated with the pathogenic bacteria. 50 µl bacteriocin supernatant were added in 5-mm agar well seeded with indicator bacteria, and after 24-hour incubation at 37°C, inhibition zones were measured to assess antimicrobial activity.

Ammonium sulphate precipitation of bacteriocin

The Cell-free supernatant (Crude bacteriocin) was precipitated using 60% ammonium sulfate. First, a 76.68% ammonium sulfate solution was prepared to create a 100% saturated solution. Then, the 60% saturated solution was prepared using a specific formula. Ammonium sulfate was slowly added with a gentle magnetic stirrer, followed by allowing it to precipitate briefly. After centrifugation at 6000 rpm at 4°C for 20 minutes, the supernatant was discarded, and the pellets were dissolved in tris-HCl buffer (pH 8.0).

Extraction of bacteriocin

The precipitated bacteriocin was further extracted with volumes of a mixture of chloroform/methanol (2/1, v/v). After 1 h at 4°C, the white precipitate formed was centrifuged for 40 min at 6000 RPM and resuspended in 3ml ultrapure water. This material was considered to be a partially purified bacteriocin.¹⁰

Molecular identification of bacteriocin

The partially purified bacteriocin were identified on a molecular basis by using SDS PAGE.¹¹

Characterization of bacteriocin

Effect of pH

Five ml aliquot of partially purified bacteriocin was taken in test tubes and the pH of the contents were adjusted to pH 2, 4, 6, 8 and 10

separately, using either diluted NaOH or HCl (1M NaOH or 1M HCl solution). After allowing the samples to stand at room temperature for 2 h, the antimicrobial activity was assayed.¹²

Effect of temperature

About 5 ml of partially purified bacteriocin in different test tubes was taken and then heated at 30, 40, 60, 90 and 121°C for 15 min under pressure. The heat treated bacteriocin samples were then assayed for antimicrobial activity.

Effect of proteolytic enzyme (bromelain)

Five ml aliquot of bacteriocin was taken in test tubes and treated with bromelain (1 mg/ml) at pH 7. The test tubes with and without the enzyme (control) were incubated at 37°C for 2 h and heated at 100°C for 3 min to denature the enzyme. Both the control and samples were assayed for antimicrobial activity by using well diffusion method.¹⁴

Effect of bacteriocin on meat preservation

Beef samples were divided into two experiments. One set was treated with bacteriocin supernatants, the other with a salt and turmeric solution as a control. After storage at room temperature, sensory analysis was conducted. The most promising bacteriocin-treated samples, based on sensory evaluation over 48 hours, were tested for Total Bacterial Count (TBC) and Coliform Bacterial Count (CC) at 0, 12, 24, and 48 hours to assess pathogen impact. The formula for calculating both total bacterial count and total coliform count was done by-

CFU/ml = (Number of colonies × dilution factor) / volume of culture plate.

Results

Isolation of bacterial strains

In this study, two yogurt samples and two milk samples were collected from local shops were collected near Khulna University. From the four samples fifteen bacterial single colonies were initially selected for further studies.

Initial screening of bacteriocin producing strain

Screening of Bacteriocin producers were carried out by agar-overlay method.¹³ In which only six isolates showed maximum zone of growth inhibition of test microorganism (*Staphylococcus spp.*) and they were selected and used for bacteriocin production studies.

Identification of bacterial isolates

For this study, six potent isolates were morphologically characterized and biochemical test were performed the test results are given below (Table 1).

Table 1 Physiological and Morphological Properties of the Selected Isolates

Morphology	Isolate Y-1.1	Isolate Y-1.2	Isolate Y-1.3	Isolate Y-1.4	Isolate M-1.1	Isolate M-1.1
Cell shape	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Colony shape	Round	Round	Round	Round	Round	Round
Colony elevation	Slightly raised	Slightly raised	Slightly raised	Slightly raised	Slightly raised	Slightly raised
Colony surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Colony color	White	White	White	White	White	White
Gram-Stain	Positive	Positive	Positive	Positive	Positive	Positive

All 6 isolates under study were found to be Gram-positive, and rod shapes were arranged in chains or pairs. Their colonies on the MRS medium were found to be circular, smooth surface, slightly raised, and

white colored. These isolates were determined as representative of the genus *Lactobacillus* (Table 2).

Table 2 Summary of Biochemical Test

Biochemical properties	Isolate Y-1.1	Isolate Y-1.2	Isolate Y-1.3	Isolate Y-1.4	Isolate M-1.1	Isolate M-1.2
Catalase	Negative	Negative	Negative	Negative	Negative	Negative
Urease	Positive	Positive	Positive	Positive	Positive	Positive
Oxidase	Negative	Negative	Negative	Negative	Negative	Negative
Citrate Utilization Test	Negative	Negative	Negative	Negative	Negative	Negative
TSI (Slant)	Red	Red	Red	Red	Red	Red
Triple Sugar Iron (TSI) TSI (Butt)	Yellow	Yellow	Red	Yellow	Yellow	Red
Gas	Negative	Negative	Negative	Negative	Negative	Negative
H ₂ S	Negative	Negative	Negative	Negative	Negative	Negative

Antimicrobial activity of bacteriocin producing strain

In the antibacterial sensitivity test, isolates Y-1.3 and M-1.2 showed no antibacterial activity. Y-1.2 and Y-1.4 inhibited all four bacteria. Y-1.1 and M-1.1 only inhibited *Staphylococcus aureus*, not *Escherichia coli*, *Pseudomonas aeruginosa*, or *Salmonella typhi*.

Partial purification of bacteriocin

The cell-free culture supernatant fluid was precipitated with 60% ammonium sulphate saturation. The bacteriocin activity of the pellicle was measured by Agar-well diffusion assay.

Extraction of bacteriocin

After chloroform/methanol extraction, the activity of the precipitate, re-dissolved in ultrapure water, was measured. This material was considered to be a partially purified bacteriocin preparation (Figure 1–3).

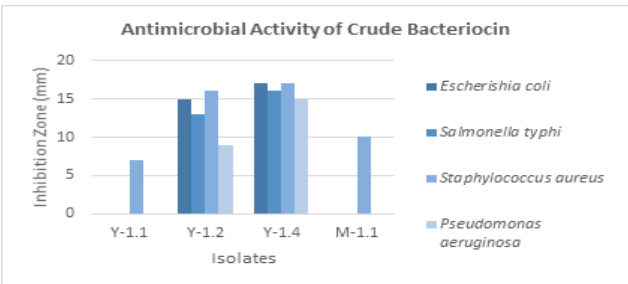


Figure 1 Antimicrobial activity of crude bacteriocins.

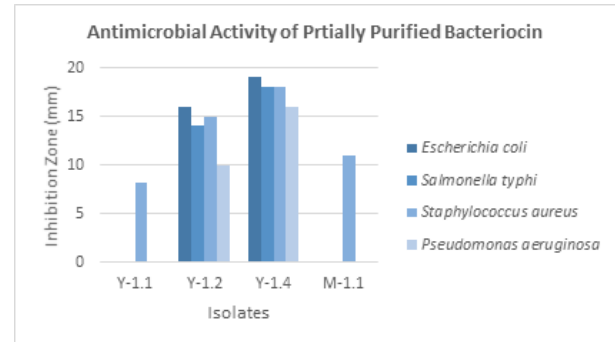


Figure 2 Antimicrobial activity of partially purified (60% ammonium sulfate) bacteriocins.

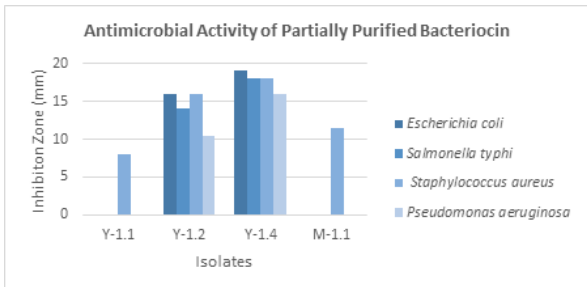


Figure 3 Activity of Partially Purified (Methanol-Chloroform) Bacteriocins.

Characterization of bacteriocin

Effect of pH

Bacteriocin showed maximum activity at pH 6.0 and remained partially active in acidic or basic conditions. Its stability across pH levels is a factor for its use in food. It retained activity in pH 3.0 to 6.0, but activity decreased from pH 8.0 to 10.0 (Figure 4).

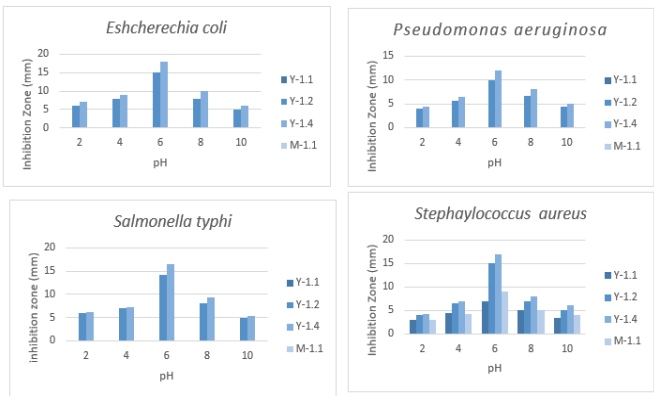


Figure 4 Effect of pH (2–10) on Bacteriocin Activity Assessed by Agar-Well Diffusion Assay Against *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Staphylococcus aureus*.

Effect of temperature

Bacteriocin activity, measured by inhibition zones against four bacteria, is thermostable up to 121°C, with some activity loss at

higher temperatures. This thermostability allows sterilization at room temperature, avoiding the need for cold storage (Figure 5).

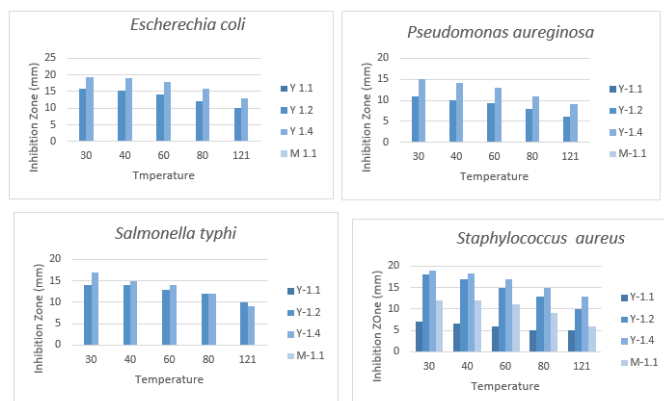


Figure 5 Effect of temperature on bacteriocin activity against test pathogens using agar-well diffusion assay.

Effect of proteolytic enzyme

Bacteriocin activity decreased with the protease enzyme bromelain at 0.25 mg/ml. When treated with protease and tested on bacteria, it showed minimal or no inhibition, indicating it's a protein that can be broken down by gastric juices, making it safe for consumption.

Effect of bacteriocin as bio-preservative on beef meat

After 12 hours, control and Y-1.1, M-1.1, Y-1.2 bacteriocin-treated beef samples changed color and emitted odor. Y-1.4 treated samples remained fresh and odorless. After 24 and 48 hours, all except Y-1.4 lost their original characteristics. Y-1.4 showed decomposition signs after 24 hours. There was no significant difference in Total Bacterial Count (TBC) at 48 hours between control and treated samples. Bacteriocin-treated samples had slightly lower TBC (3.3×10^8 CFU/g) at 24 hours compared to control (3.38×10^8 CFU/g). Antimicrobial activity decreased after 24 hours, with the lowest rate at 48 hours, where control TBC was 5×10^8 CFU/g (Figure 6).

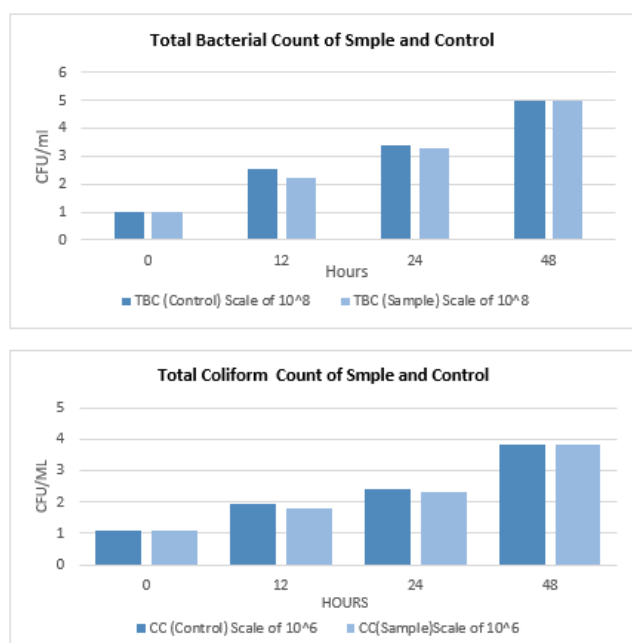


Figure 6 Coliform count of control and bacteriocin preparation on beef sample (0h-48h).

Discussion

Our study isolated six bacteriocin-producing bacteria, suspected to be *Lactobacillus* species, which showed considerable growth inhibition against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*. The bacteriocin activity was measured using various methods and showed that isolates Y-1.2 and Y-1.4 had the highest inhibition. The bacteriocin was characterized by its high heat stability, wide pH tolerance, and sensitivity to protease enzyme, suggesting it as a unique bacteriocin. The findings of this study align with those of¹⁴ and¹⁵ particularly in terms of the bacteriocin's temperature stability, pH tolerance, and sensitivity to protease enzymes.

Previous studies demonstrated that bacteriocins produced by *L. paracasei*, *L. lactis*, *L. plantarum*, and *L. pentosus* retained their activity even after being heated at 121°C for 20 minutes.¹⁶ The bacteriocin was applied on fresh beef samples to evaluate its bio-preservative potential, with isolate Y-1.4 showing better physical characteristics. The bacteriocin-treated sample showed maximum inhibition at 12 hours for both total bacterial count and coliform count. The bio-preservation activity of beef using bacteriocin yielded results that were somewhat similar to,¹⁷ which investigated the preservation of a different type of meat.

Conclusion

In summary, the bacteriocin was partially purified from *Lactobacillus* species, showing an antagonistic effect against various pathogenic bacteria, which highlights its potential as an effective preservative for different food products, thereby extending their shelf life. We aim to purify the bacteriocin further, optimizing processes to improve efficiency. Its remarkable heat stability, broad pH tolerance, and sensitivity to bromelain enzyme further emphasize its uniqueness as a bacteriocin. This indicates that bacteriocin from *Lactobacillus* species could be industrially exploited for the production of antimicrobial peptides, and additional strain improvement studies could be conducted to enhance bacteriocin production. However, extensive toxicological and acceptability tests are required before it can be approved for large-scale consumption.

Acknowledgement

Experimental facilities were provided by Food Biotechnology Laboratory, and Biochemistry and Molecular Biology Laboratory, Khulna University, Bangladesh.

Funding

This work was supported by Research and Innovation Center, Khulna University.

Conflicts of interest

The authors declare that they have no conflict of interest.

References

1. Negash AW, Tsehail BA. Current Applications of Bacteriocin. *Int J Microbiol*. 2020;2020:4374891.
2. Mokoena MP. Lactic acid bacteria and their bacteriocins: Classification, biosynthesis and applications against uropathogens: A mini-review. *Molecules*. 2017;22(8):1255.
3. Gilliland SE. Preparation and Storage of Concentrated Cultures of Lactic Streptococci. *J Dairy Sci*. 1977;60(5):805–809.

4. Luc De V and Erick JV. *Lactic acid bacteria and bacteriocins: their practical importance*. Bacteriocins of Lactic Acid Bacteria. 1994. p. 1–11.
5. Vera PE, Salvucci E, Sesma F, et al. *Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB)*. 2007.
6. De Man JC, Rogosa M, Sharpe ME. A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*. 1960;23(1):130–135.
7. Ansari A, Zohra RR, Tarar OM, et al. Screening, purification and characterization of thermostable, protease resistant Bacteriocin active against methicillin resistant *Staphylococcus aureus* (MRSA). *BMC Microbiol*. 2018;18(1):192.
8. Ahmadova A, Svetoslav DT, Yvan C, et al. Evaluation of antimicrobial activity, probiotic properties and safety of wild strain *Enterococcus faecium* AQ71 isolated from Azerbaijani Motal cheese. *Food Control*. 2013;30(2):631–641.
9. Schillinger U, Lücke FK. Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl Environ Microbiol*. 1989;55(8):1901–1906.
10. Burianek LL, Yousef AE. Solvent extraction of bacteriocins from liquid cultures. *Lett Appl Microbiol*. 2000;31(3):193–197.
11. Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*. 1970;227(5259):680–685.
12. Sharma N, Kapoor R, Gautam N, et al. Purification and Characterization of Bacteriocin Produced by *Bacillus subtilis* R75 Isolated from Fermented Chunks of Mung Bean (*Phaseolus radiates*). *Food Technology and Biotechnology*. 2011;49:169–176.
13. Motta S, Brandelli A. Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *J Appl Microbiol*. 2001;92(1):63–70.
14. Kanagaraj N, Duraisamy S, Balakrishnan S, et al. Characterization of bacteriocin producing lactic acid bacteria and its application as a food preservative. *Afr J Microbiol Res*. 2012;6(6):1138–1146.
15. Sankar NR, Priyanka VD, Reddy PS, et al. Purification and Characterization of Bacteriocin Produced by *Lactobacillus plantarum* Isolated from Cow Milk. *International Journal of Microbiological Research*. 2012;3(2):133–137.
16. Papathanasopoulos MA, Krier F, Revol AMJ, et al., “Multiple Bacteriocin Production by *Leuconostoc mesenteroides* TA33a and Other *Leuconostoc/Weissella* Strains. *Curr Microbiol*. 1997;35(6):331–335.
17. Le TTN, Long GB, Duy CN, et al. Evaluation of factors affecting antimicrobial activity of bacteriocin from *Lactobacillus plantarum* microencapsulated in alginate-gelatin capsules and its application on pork meat as a bio-preservative. *Int J Environ Res Public Health*. 2019;16(6):1017.