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ORIGINAL RESEARCH

Antibacterial Activity of Bacteriocin Like Compound Extracted from Lactic Acid Bacteria Isolated from Farm Soil, Curd, and *Gundruk*

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ABSTRACT

Objectives: This study was focused on examining the antimicrobial properties of bacteriocin like compound extracted from Lactic acid bacteria (LAB) isolates from curd, farm soil, and *Gundruk* samples.

Methods: A total of 30 samples of farm soil (10), curd (10) and *Gundruk* (10) were collected from a Kathmandu district and identified strains of LAB. Dot plate technique was used for screening of bacteriocin, then bacteriocin was extracted from precipitation method. Antimicrobial activity was done from cell-free suspension by Agar well diffusion method.

Results: In this study, 86% of LAB were isolated and identified as *Streptococcus* species (46.67%), *Lactobacillus* species (23.33%) and *Pediococcus* species (16.67%). Out of the 26 isolates, 7 isolates (23.33%) produced bacteriocin. The antibacterial activity demonstrated inhibition zones ranging from 7–16 mm for farm soil isolates, 10–20 mm for curd isolates, and 7–18 mm for *Gundruk* isolates. From mixed extraction of bacteriocin (1:1 of *Pediococcus* spp and *Streptococcus* spp), antibacterial activity was shown to all test bacteria except *S. aureus* ATCC 43300.

Conclusion: This study concluded that LAB isolates from *Gundruk* exhibited the highest antibacterial activity (18–21 mm), compared to farm soil and curd isolates, highlighting their potential as a more effective natural antimicrobial source.

Keywords: Farm soil, curd, *Gundruk*, Lactic acid bacteria and Bacteriocin

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive bacteria that produce lactic acid from various sugars through the fermentation process, which covers a wide range of health benefits (Leska, 2023). LAB is facultative anaerobic, which means that they can grow in both oxygen-rich and oxygen-poor environments, allowing them to adapt to different niches (Ganzle, 2015). LAB has a significant and extensive impact on the field of food technology. They are naturally found in fermented food and have been identified in soil, water, manure, and sewage. So, LAB are considered an important group of probiotic bacteria (Ekundayo, 2014).

LAB are present in the environment (soil), contributing to microbial diversity and the overall health of the soil ecosystem. Soils are dynamic environments with fluctuating moisture, temperature, and nutrient availability. Although LAB in soil may not be as extensively studied as fermented foods or the human microbiome, their importance in maintaining soil health and their contribution to ecological balance are increasingly recognized (Wu et al., 2021). It is found in yogurt (*Lactobacillus bulgaricus, Streptococcus thermophilus*), sauerkraut, kimchi, pickle (*Lactobacillus brevis*), cheese, raw vegetables, soil, plant materials, and honey. They are also found in milk and milk Products. They are also gut flora, which is present in the human intestine and maintains gut health (Zhong et al., 2022).

Certain strains of LAB are classified as probiotic bacteria having beneficial effects on the human digestive system. Probiotics contribute to a healthy balance of microorganisms in the gut, aid digestion, and have potential immunomodulatory and anti-inflammatory effects (Ganzle, 2015). LAB produces

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different compounds like organic acids, hydrogen peroxide, diacetyl, carbon dioxide, etc. to kill other microorganisms. Bacteriocins are also produced by LAB, which have specialized peptides having antimicrobial activity and able to specifically target similar bacterial strains or harmful bacteria (Zotta, 2017). The growing global threat of antibiotic resistance has demanded a renewed focus on research into novel antibacterial agents. Isolation and characterization of LAB for the production of antibiotics has proven to be an important attempt in the research.

Many of LAB are still poorly understood, and their potential to produce antibiotics is unknown. These LABs can produce antibiotics that help fight harmful bacteria. (Doo et al., 2024). The study of comprehensive and systematic analysis of soil bacteria with the potential to produce antibiotics was done advanced microbiological and molecular techniques (Shabana et al., 2013). There are several studies in LAB which were isolated from different samples like milk, milk products, fermentation products, soil etc. However, LAB produced secondary metabolites known as bacteriocin having antibacterial activity against pathogenic bacteria were less studied. So, the main aim of study was to extract bacteriocin like compound from Lactic Acid Bacteria (LAB) isolates from curd, farm soil and *Gundruk* samples and perform its antimicrobial activity against highly resistance bacteria. Furthermore, this study has done comparative study of LABs producing bacteriocin from different sources.

METHODS

Study design, area, site, and duration

A descriptive cross-sectional study was conducted and a total of 30 samples were collected from the Kathmandu district, including 10 samples each from farm soil, curd, and *Gundruk*. This study was done from December 2023 to April 2024.

Sample collection and processing

The Convenience sampling method was used for the sample collection. The samples were collected in sterile zip-lock bags to prevent moisture loss during transportation, and then the samples were transported to the Microbiology laboratory of Padmakanya Multiple Campus.

Isolation of Lactic Acid Bacteria

LAB were isolated on MRS (de Man Rogosa Sharpe) agar containing 1% (w/v) $CaCO_3$. One gram of each sample was suspended in 9 ml of sterile phosphate buffer and serially diluted (10^{-1} to 10^{-6}), and 1 ml of each dilution was spread on the agar plates. The plates were then incubated at 37°C for 48 hrs (*Kazemipor* et al., 2012).

Characterization of Lactic Acid Bacteria

From the pure culture of obtained LAB, the morphology of the colony was studied. They were characterized by using various techniques such as Gram staining, oxidase test, catalase test, motility test, spore staining, and fermentation test, as stated in Bergey's manual of determinative bacteriology.

Sub-culture of test bacteria

Test bacteria, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus* spp, as well as *E. coli* ATCC 25922 and *S. aureus* ATCC 43300, were obtained from the Central Department of Microbiology, Kritipur, Kathmandu and sub-cultured on Nutrient agar. The bacteria were confirmed by Gram staining and biochemical tests and pure cultures were used to evaluate the antibacterial activity of LAB.

Screening of potential Lactic Acid Bacteria for bacteriocin like compound

After characterization, isolated LAB were screened for antibacterial activity using the dot plate technique on Mueller-Hinton agar. Test bacteria *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Bacillus* species were lawn cultured on the MHA agar, and isolates of *Lactobacillus* species, *Streptococcus* species and *Pediococcus* species were point inoculated. Plates was incubated at 37°C for 24 hrs, after which the zone of inhibition were measured *(*Ma et al.*, 2019).*

Extraction of Bacteriocin like compound

Pure LAB cultures with inhibitory activity were grown in MRS broth (pH 7) at 37°C for 48 hrs. Cultures were centrifuged at 10,000 rpm for 20 min at 4°C to obtain a cell-free supernatant, then neutralized to pH 7 using 1 M NaOH. Bacteriocin was then eluted from Whatman filter paper in potassium phosphate buffer and collected in sterile test tubes *(*Yang et al.*, 1992).*

Determination of antibacterial activity of Bacteriocin like compound by agar well diffusion method

To determine antimicrobial activity, test bacteria were grown in nutrient broth for 4 hrs at 37°C and adjusted to 0.5 McFarland standard. Bacterial suspensions were

swabbed onto Mueller-Hinton agar, and 6 mm wells were made. A 70 µl solution of bacteriocin from soil, curd, and *Gundruk*, along with sterile water (negative control) and ciprofloxacin (positive control) were added to the wells and allowed to diffuse for 15 min. Plates were incubated at 37°C for 24–48 hrs, after which the zones of inhibition were measured *(Zhennai, 2000)*.

Quality control in the laboratory

Quality was also monitored for each laboratory equipment's throughout the study period. Standard culture of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 43300) were used for the interpretation of result in antimicrobial activity.

Data Analysis

All the data was obtained from this study was entered into Microsoft Excel 2016 and analyzed by percentage calculation.

RESULTS

In this study, among 30 samples (farm soil, curd and *Gundruk*), 26 (86.67%) isolates were identified as LAB.

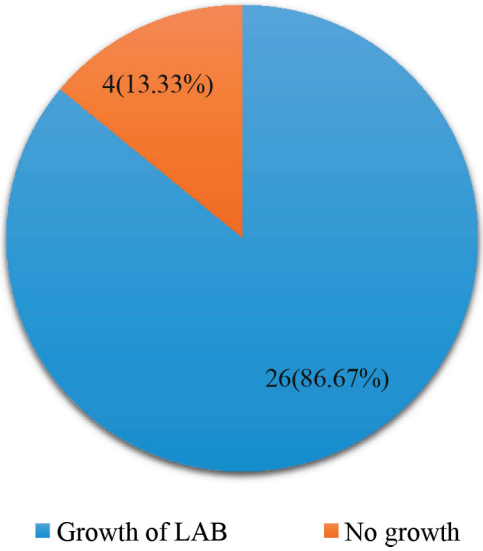


Figure 1: Growth of LAB among total samples

Types of Lactic Acid Bacteria isolated among total samples

Among 26 (86.67%) LAB isolates, 14 (46.67%) *Streptococcus* spp were identified followed by 7(23.33%) *Lactobacillus* spp and 5 (16.67%) *Pediococcus* spp from farm soil, curd, and *Gundruk* samples.

Table 1: Types of Lactic Acid Bacteria isolated among the total samples

Samples	Number of samples	*Lactobacillus* spp N (%)	*Streptococcus* spp N (%)	*Pediococcus* spp N (%)	Total N (%)
Farm Soil	10	4 (13.33)	4 (13.33)	2 (6.67)	10(33.33)
Curd	10	3 (10.0)	5 (16.67)	0	8(26.67)
Gundruk	10	0	5 (16.67)	3 (10.00)	8(26.67)
Total	**30**	**7(23.33)**	**14(46.67)**	**5(16.67)**	**26(86.67)**

N = Number, % = Percentage

Carbohydrate Fermentation test of Lactic Acid Bacteria

In carbohydrates fermentation test, LAB isolates from farm soil, curd and *Gundruk* showed fermentation to different sugars including glucose, lactose, sucrose and fructose.

Table 2: Fermentation test of different carbohydrates by Lactic Acid Bacteria isolates of farm soil samples

Carbohydrates	*Lactobacillus* species (4)	*Streptococcus* species (4)	*Pediococcus* species (2)
Glucose	+	+	-
Lactose	+	+	+
Sucrose	+	-	+
Fructose	+	+	+

+ = Fermenter, - =Non-fermenter

Table 3: Fermentation test of different carbohydrates by Lactic Acid Bacteria isolates of curd samples

Carbohydrates	*Lactobacillus* species (3)	*Streptococcus* species (5)
Glucose	+	+
Lactose	+	+
Sucrose	+	-
Fructose	+	+

+ = Fermenter, - = Non-fermenter

Table 4: Fermentation test of different carbohydrates by Lactic Acid Bacteria isolates of *Gundruk* samples

Carbohydrates	*Streptococcus* species (5)	*Pediococcus* species(3)
Glucose	+	+
Lactose	+	-
Sucrose	-	+
Fructose	+	+

+ = Fermenter, - = Non-fermenter

Screening of potential Lactic Acid Bacteria for bacteriocin like compound

A total of 26 LAB isolates were screening for bacteriocin against test bacteria in which 7 LAB isolates were positive against all test bacteria.

Table 5: Screening of potential Lactic Acid Bacteria for bacteriocin like compound

Test bacteria	Farm soil isolates			Curd isolates		Gundruk isolates	
	S1	S2	S3	C1	C2	G1	G2
Gram positive bacteria							
S. aureus ATCC 43300	6mm	5mm	10mm	10mm	8mm	5mm	-
S. aureus	7mm	8mm	15mm	8mm	10mm	8mm	-
Bacillus spp	-	6mm	12mm	8mm	-	-	-
Gram negative bacteria							
E. coli ATCC 25922	6mm	6mm	9mm	-	12mm	8mm	7mm
E. coli	10mm	7mm	11mm	10mm	-	7mm	10mm
K. pneumoniae	-	9mm	9mm	6mm	9mm	5mm	-
P. aeruginosa	-	4mm	6mm	-	-	-	9mm

- = no zone of inhibition, S1 = *Lactobacillus* spp, S2 = *Streptococcus* spp, S3 = *Pediococcus* spp C1= *Lactobacillus* spp, C2 = Streptococcus spp, G1 = *Streptococcus* spp, G2 = *Pediococcus* spp

Antibacterial activity of bacteriocin like compound against test bacteria

In this study, bacteriocin like compound extracted from farm soil, curd and *Gundruk* showed different zone of inhibition ranged from 7mm to 20mm against all test bacteria.

Table 6: Antibacterial activity of bacteriocin like compound against test bacteria

Test bacteria	Soil isolates			Curd isolates		*Gundruk* isolates	
	(S1)	(S2)	(S3)	(C1)	(C2)	(G1)	(G2)
Gram positive bacteria							
S. aureus ATCC 43300	-	-	-	-	-	-	-
S. aureus	-	-	-	-	-	-	7mm
Bacillus spp	-	-	-	15mm	13mm	18mm	15mm
Gram negative bacteria							
E. coli ATCC 25922	7mm	-	11mm	12mm	10mm	13mm	12mm
E. coli	13mm	16mm	9mm	20mm	16mm	17mm	15mm
P. aeruginosa	-	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-	-

- = No zone of inhibition, S1 = *Lactobacillus* spp, S2 = *Streptococcus* spp, S3=*Pediococcus* spp, C1= *Lactobacillus* spp, C2=*Streptococcus* spp, G1= *Streptococcus* spp and G2= *Pediococcus* spp.

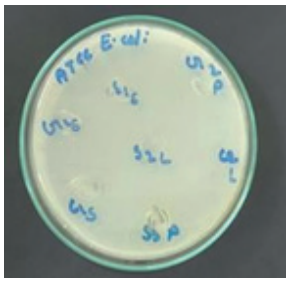
Antibacterial activity of mixed bacteriocin like compound against test bacteria

In this study, mixed bacteriocin like compound (1:1) from farm soil (S1, S2 & S3), curd (C1 &C2) and *Gundruk* (G1 & G2) showed antibacterial activity against all test bacteria except *S. aureus* ATCC 43300.

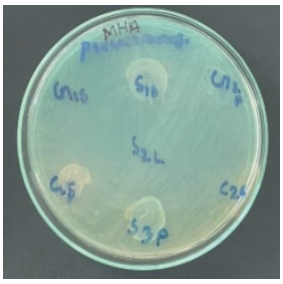
Table 7: Antibacterial activity of mixed bacteriocin like compound against test bacteria

Test Bacteria	Farm Soil (S1, S2 & S3)	Curd (C1 &C2)	*Gundruk* (G1 & G2)
Gram positive bacteria			
S. aureus ATCC 43300	-	-	-
S. aureus	-	-	10mm
Bacillus spp	10mm	20mm	18mm
Gram negative bacteria			
E. coli ATCC 25922	11mm	19mm	21mm
E. coli	15mm	20mm	19mm
P. aeruginosa	-	17mm	18mm
K. pneumoniae	11mm	10mm	15mm

- = No zone of inhibition, S1 = *Lactobacillus* spp, S2 = *Streptococcus* spp, S3 = *Pediococcus* spp, C1 = *Lactobacillus* spp, C2 = *Streptococcus* spp, G1 = *Streptococcus* spp and G2 = *Pediococcus* spp

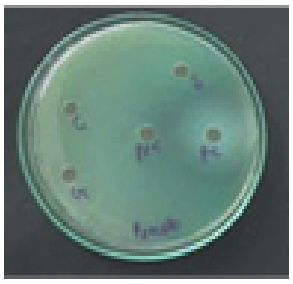


(A)

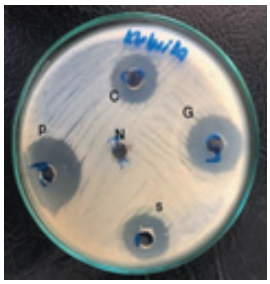


(B)

Screening of *Lactic Acid Bacteria* for bacteriocin extraction against (A) *Escherichia coli* ATCC 252922 and (B) *Pseudomonas aeruginosa* by dot plate method in MHA plate



(C)



(D)

Antibacterial activity of mixed bacteriocin extraction (1:1) from *Lactic Acid Bacteria* against *Pseudomonas aeruginosa* (C) and *Klebsiella pneumonia* (D) in MHA plate

DISCUSSION

In the present study, 26 (86.67%) isolates were identified as LAB in which three LAB isolates were capable of producing bacteriocin from *Gundruk*, farm soil, and curd. By comparing the morphological, physiological and biochemical tests, the isolates were identified as 14 (46.67%) *Streptococcus* spp, 7(23.33%) *Lactobacillus* spp (23.33%) and 5 (16.67%) *Pediococcus* spp In the study of Diop et al., (2007), 12 strains of LAB that produce bacteriocin were isolated in fermented foods. Ekundayo (2014) isolated 17 isolates of LAB in which 11 isolates were identified as *Lactobacillus* spp The result of the present study is in accordance with the report of Galvez et al., (2007) who revealed that members of LAB could be detected in a variety of habitats including fermented foods.

The isolates of LAB were screened against Gram positive bacteria (*S. aureus*, *S. aureus* ATCC 43300 and *Bacillus* species) and Gram negative bacteria (*E. coli* ATCC 25922, *E. coli*, *P. aeruginosa* and *K. pneumoniae*) by using dot plate technique on MHA plate. In this study, dot plate technique showed the zone of inhibition ranges from 5mm to 15mm against test bacteria. Among 7 LAB isolates, antibacterial activity showed against *E. coli*, *K. pneumoniae* and *S. aureus* but did not show any antibacterial property against *P. aeruginosa* and *Bacillus* species. Similarly, Boguta, et al., (2014) screened 296 strains of *Lactobacillus* and *Pediococcus*.

From farm soil samples, bacteriocin like compound extracted from S1, S2 and S3 samples showed antibacterial activity against Gram negative bacteria *E. coli* ATCC 25922 by 7mm and 11mm and *E. coli*. by 13mm, 6mm and 9mm of zone of inhibition respectively. Similarly, bacteriocin like compound extracted from C1 and C2 showed inhibitory action against Gram positive bacteria *Bacillus* species by 15mm and 13mm of zone of inhibition respectively and Gram negative bacteria *Escherichia coli* ATCC 25922 by 12mm and 10mm of zone

of inhibition respectively and *Escherichia coli* by 20mm and 16mm of zone of inhibition respectively. From *Gundruk* samples, G1 and G2 showed antibacterial activity against Gram positive bacteria *S. aureus* and *Bacillus* species by 7mm and 15mm of zone of inhibition respectively and Gram negative bacteria *Escherichia coli* and *Escherichia coli* ATCC 25922 by 17mm and 15mm of zone of inhibition respectively. Similarly, Collins, et al., (1983) reported that the antibacterial activity of LAB strains against *S. aureus* and *P. fragi*. Elayaraja, et al., (2014) showed bacteriocin as inhibition activity against pathogens and concluded that LAB showed antimicrobial activities of wide range.

Further, bacteriocin like compound extracted from LAB isolates of farm soil (S_1, S_2 and S_3), curd (C_1 and C_2) and *Gundruk* (G_1 and G_2) were mixed (1:1) to analyze the inhibitory activity. The bacteriocin extracted from farm soil was able to inhibit *E. coli* ATCC 25922 (11mm), *E. coli* (15mm), *K. pneumoniae* (11mm) and *Bacillus* species (10mm) with respect to the diameter of inhibition zone. The bacteriocin extracted from curd was able to inhibit *E. coli* ATCC 25922 (19mm), *E. coli* (20mm), *P. aeruginosa* (17mm), *K. pneumoniae* (10mm) and *Bacillus* species (20mm) with respect to the diameter of inhibition zone. The bacteriocin extracted from *Gundruk* was able to inhibit *S. aureus* (10mm), *E. coli* ATCC 25922 (11mm), *E. coli* (15mm), *P. aeruginosa* (18mm), *K. pneumoniae* (11mm), and *Bacillus* species (10mm) with respect to the diameter of the inhibition zone. However, the bacteriocin like compound extracted from LAB isolates of *Gundruk*, curd and farm soil samples in mixed ratio showed the higher zone of inhibition. Bacteriocins like compound extracted from these isolates exhibited varying antibacterial activity, in which bacteriocins like compound extracted from *Gundruk* samples showed the strongest effects.

Combining bacteriocins like compound from different sources in a mixed ratio (1:1:1) increased their antibacterial spectrum, affecting the highest number of test bacteria. However, bacteriocin extracted from all samples did not showed zone of inhibition to *S. aureus* ATCC 43300. Similar result was reported by Sharma et al., (2021), the antimicrobial activity of bacteriocin like compound against *Bacillus* spp, *Shigella* spp and *E. coli* and but didn't show inhibition to *Salmonella* spp, *S. aureus* and *K. pneumoniae*. Perez, et al., (2014) concluded that bacteriocins like compound also use as a next generation antibiotics for inhibiting the multi drug resistant bacteria. However, this study showed antibacterial activity of bacteriocin like compound in limitation number of samples. So, further study can be done in different potential species for the development of novel antibiotic.

CONCLUSION

Diverse species of lactic acid bacteria were isolated from farm soil, curd and *Gundruk* samples however, very few species were able to produce bacteriocin. Bacteriocin extracted from them showed different zone of inhibition to all test bacteria. Overall, this study highlight LAB producing natural antimicrobial agents. So, in this today's world, research on antimicrobial activity of bacteriocin like compound extracted from LAB become a great importance for next generation antibiotics.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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