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ORIGINAL RESEARCH

Detection of Antimicrobial Activities and Bacteriocin Encoding Genes in Lactic Acid Bacteria Isolated from Fermented Foods in Kirtipur

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ABSTRACT

Objectives: The objective of this study was to isolate and identify Lactic Acid Bacteria (LAB) from fermented foods and evaluate their antimicrobial activities as well as to detect bacteriocin encoding gene and also determine probiotic effect of selective LAB isolates.

Methods: A total of 26 fermented food samples were collected from different small scale dairy shops and community of Kirtipur, Kathmandu and LAB were isolated following standard plate count method. Preliminary identification of isolates was done by staining and biochemical tests. The genes encoding specific bacteriocin were detected using polymerase chain reaction.

Results: Based on the morphological, microscopy and biochemical characteristics, a total of 40 LAB were screened; among them 25 (62.5%) were Gram-positive rod shape LAB A (*Lactobacillus* spp) type and 15 (37.5%) isolates were gram-positive cocci shape LAB B type. Among 40 isolates of Lactic Acid Bacteria, cell free supernatant of 24 (60%) showed antibacterial activity with highest being that of isolates Ca3 and Ca4 LAB against all 6 test bacteria. Isolated LAB were most effective against *Pseudomonas aeruginosa* i.e. out of 24 LAB 20 (83.33%) isolates showed inhibition zone. Enterocin gene was detected in 4 (16.66%) LAB isolates while nisin and pediocin genes were not detected in isolated LAB. All 9 selected LAB isolates were able to resist high acidic condition at pH 3 till 24 hrs, 0.5% and 1% bile salt concentration while isolates C3 (*Enterococcus* spp) and Ca3 (*Lactobacillus* spp) were able to resist pH 3 till 48 hrs of incubation. Therefore, they can survive in stomach high acidic and bile condition indicating their probiotic potentiality.

Conclusion: These findings emphasize LAB's potential as natural antibacterial agents that could be exploited in food preservation and as probiotics to improve human health.

Keywords: LAB, Antibacterial activity, Bacteriocin, Probiotics, Fermented foods

INTRODUCTION

Fermentation of food is one of the oldest processing and preserving methods used all around the world. Varieties of fermented foods has been included in Nepali diet from prehistory. Most commonly used fermented foods are *Dahi, Gundruk, Achaar, Kinema*, although uses of fermented foods vary according to community. These fermented foods harbor wide variety of microorganisms including LAB. LAB is employed for preservation of fermented foods. LAB mainly produce lactic acid as their by-product during their metabolic activities. They play important role in agricultural and

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clinical sectors and also possess therapeutic properties that are vital for human health enhancement (Tamang et al., 2005).

Lactic Acid Bacteria produces different bacteriocins which can be used for the preservation of food and is also safer than other antimicrobials (Clevland et al., 2001). Bacteriocin are ribosomally synthesized, by diverse bacterial groups including LAB, which is biologically antimicrobial in nature, while chemical structure is composed of peptides and proteins (Cotter et al., 2005). Bacteriocins produced by LAB are considerably safe, that's why gaining more attraction on different application. LAB bacteriocin inhibits the growth of closely related bacteria at low concentration in nanomolar level with no side effects on public health which leads to less chances of development of antimicrobial resistance. Bacteriocin acts against the target cell membrane by making a quick pore at very low concentration. Due to its degradable nature in human digestive system, it cannot remain in outer environment also (Zendo et al., 2013).

Probiotics are live microbial food supplements that beneficially affect the host by improving intestinal microbial balance. According to the FAO/WHO (2002), probiotics are defined as "live

microorganisms which, when administered in adequate amounts, confer a health benefit on the host." Most probiotics are bacteria, primarily LAB, though some yeasts (e.g., *Saccharomyces* spp) and molds (e.g., *Aspergillus* spp) are also used. Common probiotic genera include *Lactobacillus, Bifidobacterium, Lactococcus, Enterococcus*, and *Bacillus* (Peng et al., 2022).

The current study has been designed to detect and characterize the anti-bacterial activities, probiotic activities, presence of bacteriocin encoding genes, in lactic acid bacteria isolated from fermented foods used in Nepal.

METHODS

Samples

A total of 26 fermented food (13 fermented dairy product and 13 fermented vegetable products) were collected from Kirtipur from July 2024 to December 2024. Fermented dairy products included yoghurt and cheese while fermented vegetables product included raddish pickle, *Tama*, and *Gundruk*. All of the samples were selected and collected following convenience sampling method, in sterile zip-locked bag and were brought to Central Department of Microbiology, TU, Kirtipur, within 3 hrsof collection, maintaining reverse cold chain temperature (2-8^{0}C). Then samples were further processed for isolation of the lactic acid producing bacteria (LAB) (Bhattrai et al., 2016).

Isolation of LAB

For isolation of LAB, 1gm of each sample (*Gundruk, Tama*, raddish pickle, Yogurt and Cheese) was added to 9ml of phosphate buffer saline to make 1/10 dilution and mixed properly. The 1/10 dilution was then serially diluted up to 10^{-6} dilution. The samples from 10^{-2}, 10^{-4}, 10^{-6} dilution were pipetted out in the Petriplate. Then, de Man, Rogosa and Sharpe (MRS) media incorporated with 1% calcium carbonate was poured properly and incubated at 37^{0}C for 48 h. The isolated colonies with clear zone were sub-cultured on MRS agar.

Preliminary identification was done based on colony morphology on MRS agar, Gram-staining and spore staining. Further confirmation of the isolates were done based on biochemical test (Catalase test, oxidase test, motility test, MR-VP test, TSIA test and citrate utilization tests), sugar fermentation (Glucose, Lactose, Maltose and Sucrose) test results, growth at different range of pH and temperature.

Extraction of cell free supernatant from LAB isolates

The LAB isolates were grown in MRS broth for 48 h at 37^{0}C. Then centrifugation of broth was done at 8000 rpm for 10 minutes. The obtained cell free supernatant (CFS) was further processed for screening of antimicrobial activity.

Antimicrobial activity of cell free supernatant of LAB isolates against test organisms

Five ATCC cultures including *P. aeruginosa* 49619, *S. aureus* 29213, *S. aureus* 43300, *E. coli* 25922, *Klebsiella* spp 700603 and one food borne pathogen *Salmonella* spp isolated from meat sample in the department were used as test organisms for determination of the antibacterial activity of the cell free supernatant from the isolated LAB. Briefly, wells were made on Muller Hinton Agar (MHA) media with the cork-borer of 6mm diameter and test organisms were carpet cultured on MHA. Cell free LAB extract (50 µl) and control (PBS) were loaded on the well and allowed to diffuse at room temperature

for 30 minutes. The loaded plates were incubated at 37^{0}C for 24 hrs and zone of inhibition were observed and recorded (Abdel Tawab et al., 2023).

Probiotic test of selective isolates

Selective isolates with inhibition zones (larger than 16 mm) against the standard ATCC test organisms (*P. aeruginosa* 49619, *S. aureus* 29213, *S. aureus* 43300, *E. coli* 25922, *Klebsiella* spp 700603) were further tested for four different probiotic activities including bile salt tolerance, sodium chloride (NaCl) tolerance, growth on different range of pH and temperature. For bile salt and NaCl tolerance test, total of 9 selected isolates were grown in MRS broth incorporating different concentration (0.5%, 1%, 1.5% and 2% of bile salt) for bile salt tolerance test; and 2% and 4% for NaCl tolerance test; Optical Density (OD) of the incubated tubes were measured using ELISA reader after 24 hrs of incubation at 37^{0}C.

For pH test two acidic pH 3 and 4.4 of MRS broth were adjusted by using Hydrochloric acid (HCl), Then isolates were inoculated in different pH adjusted MRS broth and OD were measured at different incubation time i.e. 0 hr, 24 hrs and 48 hrs (Khushboo et al., 2023). Similarly for temperature, isolates in MRS broth were incubated at different temperature i.e. 28^{0}C, 37^{0}C and 45^{0}C and OD were measured after 24 hrs of incubation at 37^{0}C.

Detection of bacteriocin gene by polymerase chain reaction (PCR)

Bacterial DNA extraction

LAB which showed inhibitory effect against test organism were only proceeded for molecular detection of selected bacteriocin gene. Fresh bacterial LAB culture was used to extract bacterial DNA by phenol-chloroform method. Quantification of Bacterial DNA and quality assessment were performed using nanodrop (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Conventional Polymerase Chain reaction (PCR) to detect Bacteriocin gene

Following the manufacturer's instruction, PCR was used to detect the presence of bacteriocin encoding gene. Briefly, 2 µL of DNA template, 5 µL master-mix (Solis BioDyne), 0.5 µL each of forward and reverse primers, and 17 µL nuclease-free water were added making total volume of reaction mixture 25 µL. Three different reverse and forward primers for Pediocin, Enterocin and Nisin were used to detect bacteriocin gene. The following steps were part of PCR program: 30 cycles of 94^{o}C for 5 min, 94^{o}C for 1 min, 55^{o}C for 30 Sec, 72^{o}C for 45 Sec followed by 72^{o}C for 6 min (Fuente-salcido et al., 2015).

Table 1. Primers used for PCR

Target gene	Primer sequence	PCR product size (bp)	Reference
Pediocin (*ped*)	Forward 5'-GGTAAGGCTACCACTTGCAT-3' Reverse 5'-GGGTACCACTCATAGTGGAA-3	332	Suwanjinda et al., 2007
Enterocin (*entA*)	Forward 5'-GGGTACCACTCATAGTGGAA-3' Reverse 5'-CCAGCAGTTCTTCCAATTTCA-3'	412	
Nisin (*nisR*)	Forward 5'-CTATGAAGTTGCGACGCATCA-3' Reverse 5'-CATGCCACTGATACCCAAGT-3'	608	

Analysis of PCR Results

Amplified PCR products were run on 1.5% agarose gel and the bands were observed on Gel documentation system.

RESULTS

Isolation and Identification of lactic acid bacteria

From 26 samples comprising fermented dairy and fermented vegetable product, 40 LAB isolates were screened based on clear zone around colonies. Among them 25 (62.5%) isolates were gram-positive, non-spore forming rod shaped (LAB A) type and 15 (37.5%) isolates were gram-positive cocci shaped (LAB B) Type. These bacteria fermented all four sugars used and only LAB B type shown growth at 45 ^{o}C.

Distribution of Lactic Acid Bacteria according to samples type

Out of 26 samples collected, 24 samples showed presence of LAB. Among 40 LAB isolates 21(52.5%) were screened from fermented vegetables while 19 (47.5%) were from fermented dairy products ($p > 0.05$).

Distribution of Lactic Acid Bacteria according to sample age

Among 40 LAB isolates, 12 (30%) LAB isolates were from 1-3 days old samples, 15 (37.5%) LAB isolates were from 1-3 weeks old sample and 13 (32.5%) LAB isolates were from more than one month old samples

($p > 0.05$).

Antibacterial activities of cell free extract of LAB isolates against test organisms

Out of total 40 LAB isolates, extract from only 24 (60%) isolates showed inhibition zone against different test organisms. The zone of inhibition ranged between 7 to 28 mm in diameter with maximum being shown by extract from isolate T2. Among these 24 isolates 16 were rod and 8 were cocci.

Probiotic attributes of isolated LAB

Acid tolerance test

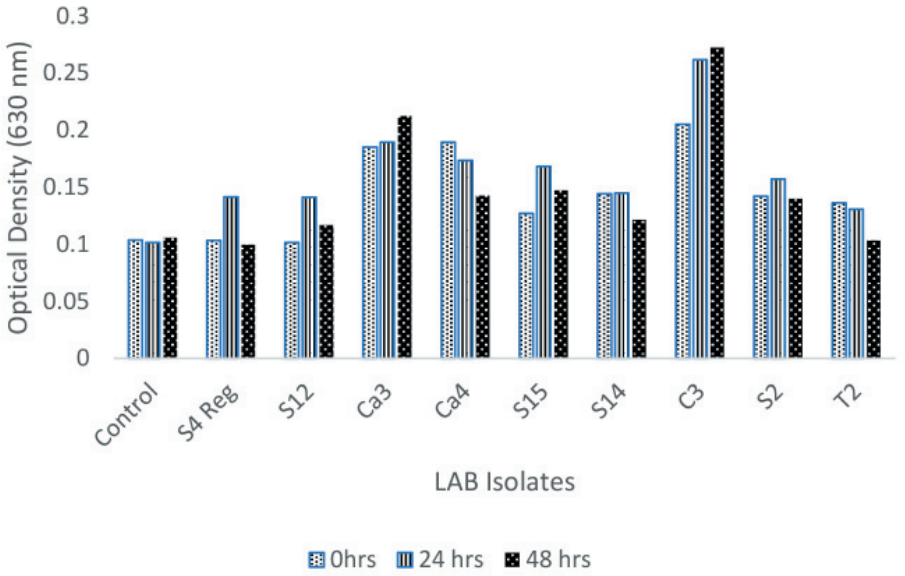

Figure 1: Acid tolerance test of LAB isolates at pH 3

From 24 LAB isolates with inhibitory effect on test organisms, only 9 LAB isolates were selected for probiotic potentiality based on spectrum of antibacterial activity. Two isolates Ca3 and C3 showed increment in OD value at pH 3 till 48 h of incubation.

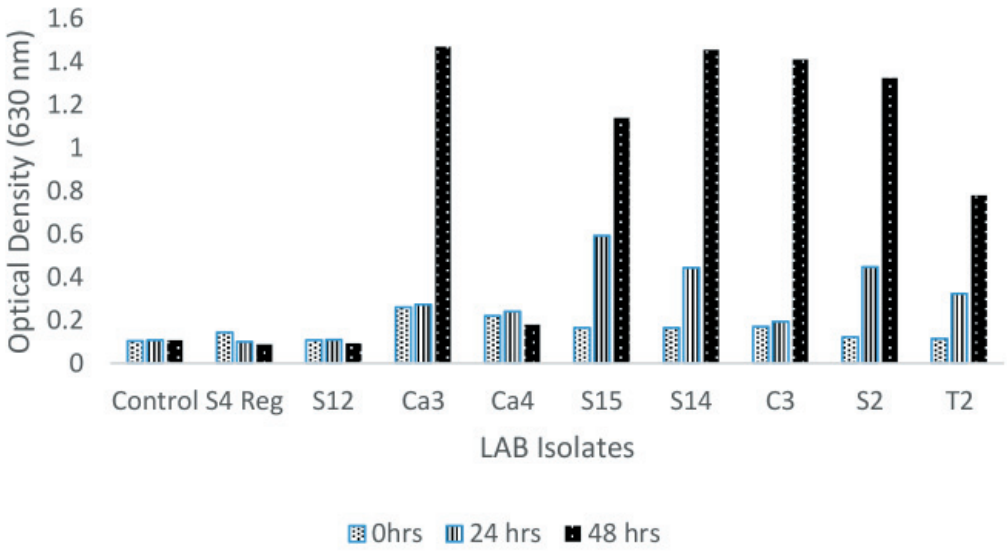

Figure 2: Acid tolerance test of LAB isolates at pH 4.4

In figure 2, LAB isolates Ca3, S15, S14, C3 and T2 showed luxurious growth till 48 h of incubation indicating their potential to tolerate low pH. While Isolates S4 Reg and S12 were unable to resist ph4.4

Bile salt tolerance test

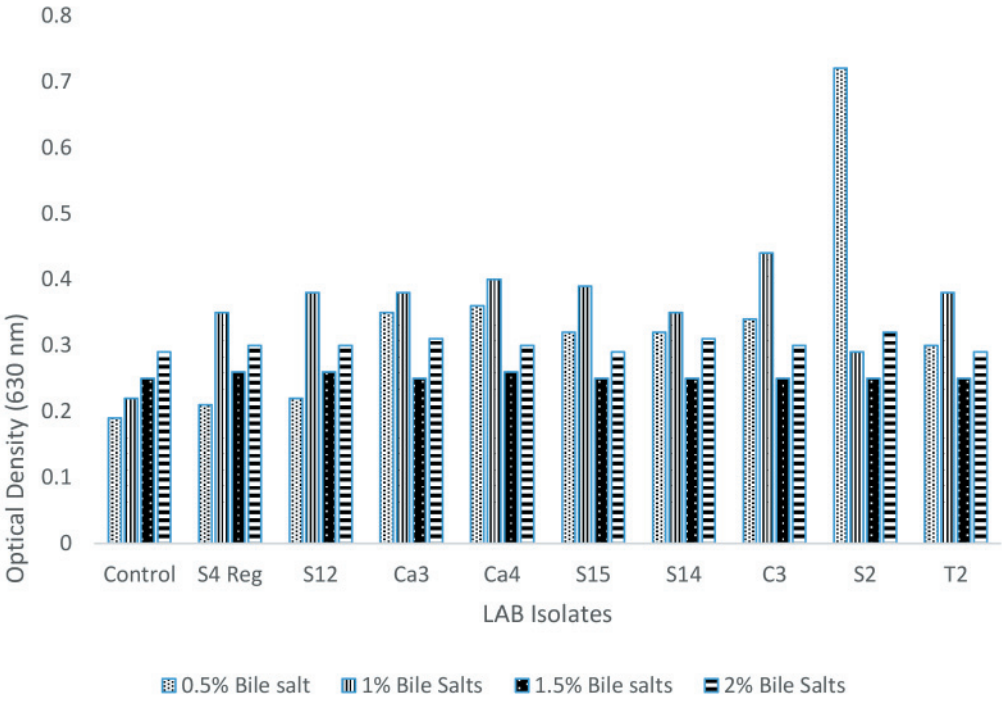

Figure 3: Bile salt tolerance test of LAB Isolates at different concentration of bile salt

Fig. 3 shows the bile salt tolerance pattern of LAB isolates after 24 h of incubation. The results from above figure clearly indicated that all of the 9 LAB isolates survived upto 0.5% and 1% bile bile salt concentration while are unable to survive in higher concentration (1.5% and 2%) of bile salts.

Growth pattern of isolated LAB at various temperature

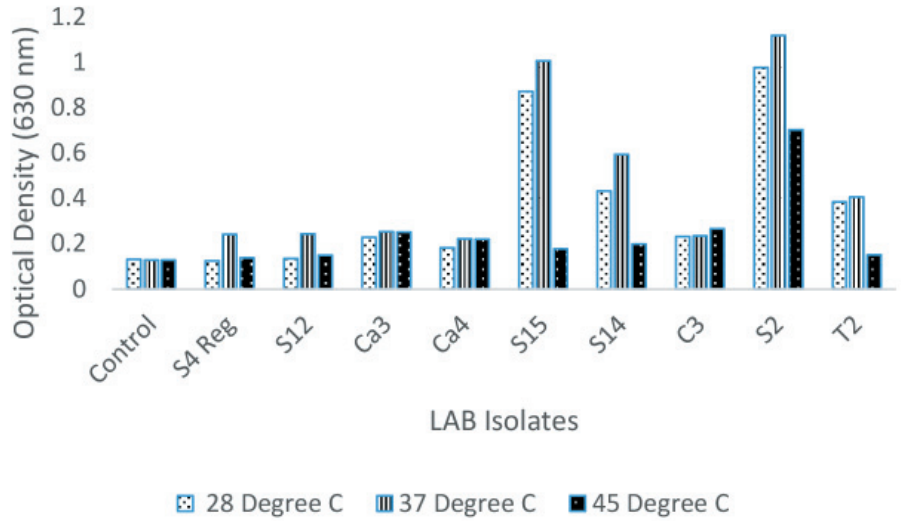

Figure 4: Growth pattern of LAB Isolates at different temperature after 24 h incubation

Figure 4 indicates growth pattern of LAB isolates at three temperature i.e. 28 °C, 37 °C and 45 °C. Most of the isolates showed optimum growth at 37 °C whereas growth of isolate S14, S15 and S2 favored at 28 °C while only isolate C3 showed optimum growth at 45 °C.

Salt (Sodium Chloride) tolerance test

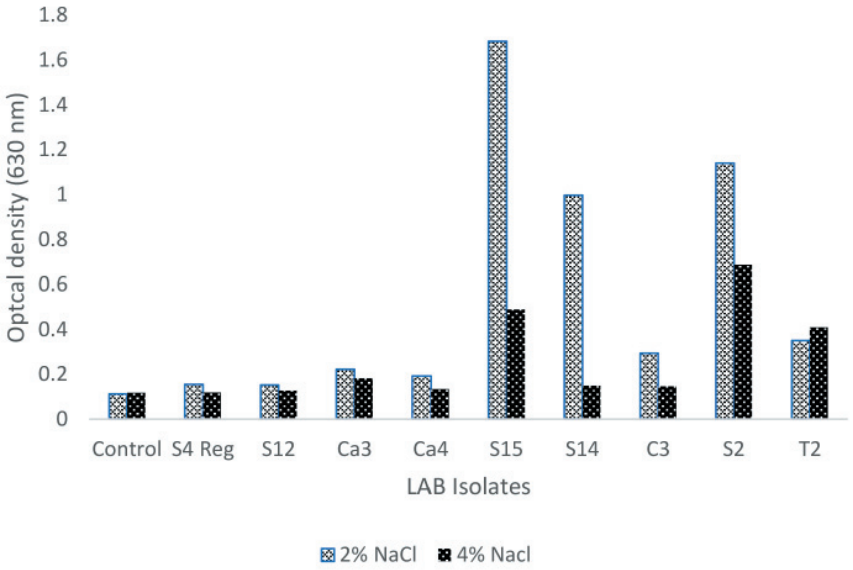

Figure 5. Salt tolerance test at different concentration of NaCl

Figure 5 represents tolerance pattern of isolates towards different concentration of NaCl. Among 9 Isolates, all of the isolates were able to grow at provided salt concentration. Whereas isolate S15, S14 and S2 depicted increment in OD value.

Detection of Bacteriocin Encoding gene (Enterocin, Nisin and Pediocin)

Among 24 isolates of LAB which showed antibacterial properties against test organisms; only 4 (16.66%) isolates showed positive result for enterocin gene. Other two bacteriocin encoding gene i. e. Nisin and Pediocin were not detected in isolates. Enterocin gene were detected in four LAB isolates i.e S_1 1.3, S_{13} 1.2, C3 and Ga.

DISCUSSION

This study aimed to screen LAB from fermented foods with antibacterial activity against proven pathogenic bacteria and detecting selected genes in potent isolates responsible for bacteriocin production. Furthermore, it also investigated on some of the probiotic properties of selected isolates. In total 26 samples were collected; only 24 samples showed presence of Lactic Acid Bacteria. For preliminary identification of isolates; Gram's staining, spore staining, biochemical test and sugar fermentation pattern were done. Among 40 isolates, 25 (62.5%) isolates were Gram positive, non-sporing rod, catalase negative, oxidase negative, and fermented all four sugars tested hence identified as *Lactobacillus* spp The remaining (37.5%) isolates were Gram positive, cocci in short chain, catalase and oxidase negative and fermented all of the four sugars used and shown growth at 45°C. The study conducted in fermented dairy products by Refay et al., 2020 reported 51(75%) rod-shaped, whereas 17 isolates (25%) were cocci shaped LAB.

Among 40 isolates of LAB, cell free supernatant of 24 (60%) showed antibacterial effect against test organisms. The CFS from some isolates i.e Ca3 and Ca4 showed inhibitory effect against all test organisms including *salmonella* spp isolated from meat. Most of the LAB isolates showed antibacterial activity against both Gram positive and Gram-negative bacteria used as test organism. The highest zone of inhibition (28 mm in diameter) was shown by CFS from T2 against *P. aeruginosa* 49619. The study conducted by Adeyemo et al., 2018 was also close to the present study where species of *Lactobacillus* showed inhibition zone against *P. aeruginosa* (20 mm, 15 mm, 19 mm and 4.5 mm) and *S. aureus* (22 mm, 16mm, 19mm and 5 mm). In similar study conducted by Adhikari et a, 2025 revealed the zone of inhibition ≥ 15 mm by extract from *Lactobacillus* spp isolated from Nepali fermented foods.

Most probiotics belongs to lactic acid bacteria (LAB). LAB's capacity for fermentation and preservation makes them extremely valuable to the food sector. Probiotic LAB has several health benefits, including modulating the immune system, gut bacteria regulation, antibacterial, cancer fighting, and anti-obesity properties. LAB are utilized as ingredients in functional foods because of their many advantages (Sadiq, 2022).

In present study, 9 LAB isolates with antibacterial activity were selected for screening of probiotic

potential. Probiotic potential was measured through acid and bile tolerance test with varying concentration as these are the major components of gastrointestinal tract hence organisms must resist these hurdles. Out of 9 LAB isolates all isolates resisted pH 3 till 24 hrs, only two isolates (Ca3, C3) resisted pH 3 till 48 hrs. LAB Isolates Ca3 and C3 have potential to be a probiotic as these isolates demonstrated antibacterial activity against test organisms and resist both pH 3 and 4.4 till 48 hrs. Among 9 LAB isolates, all of the candidates survived upto 0.5% and 1% bile salt concentration however some isolates were unable to survive in higher concentration (1.5% and 2%) of bile salts. Among the isolates, 3 were able to survive 2% and 4% NaCl. Amenu and Bacha, 2023 reported that out of 125 possible probiotic LAB isolates, 17 (13.60%) resisted 0.3 % bile salts and low pH 2, 2.5 and 3. A study reported 60 LAB isolates from colostrum of Jordanian camels. *Enterococcus faecium* and *Lactobacillus salivarius* were found to be dominating species. All 60 isolates demonstrated tolerance to varying pH (2,3,4,5,6,7,8,9,10) and additionally, the isolates as a whole exhibited varying bile resistance (%) and tolerance to varying bile salt concentrations (0.2, 0.4, 0.6, 0.8, 1, 2, and 3). Due to the unique characteristics of LAB, they considerably vary level of acid and bile salt tolerance (Pitino et al., 2012).

Antimicrobial-resistant bacteria have been increasingly reported in both clinical and community settings, with multidrug-resistant *Escherichia coli* and other resistant pathogens posing major challenges for effective treatment due to limited therapeutic options (Acharya et al., 2024; Dahal et al., 2025; KC et al., 2019; Pandit et al., 2024; Shrestha et al., 2019; Tiwari et al., 2024). This alarming trend highlights the urgent need to identify and develop novel antibacterial products, such as bacteriocin-producing lactic acid bacteria and other bioactive agents, to counteract resistance and improve public health outcomes.

LAB isolates which showed inhibitory activity against test organisms are subjected to PCR for three bacteriocin encoding gene detection. For bacteriocin encoding gene detection, specific primer sequences used in PCR was taken from as per studies done by Fuente-salcido et al., 2025. In present study 4 (16.66%) LAB isolates showed positive result for enterocin gene in PCR while other two nisin and pediocin gene were not detected in isolated LAB although these isolates demonstrated antibacterial activities against different test bacteria.

CONCLUSION

The purpose of this study was to assess the antibacterial activities and detection of bacteriocin encoding gene in isolated LAB isolated from fermented food. The samples were dominated mostly by *Lactobacillus* spp as compared to cocci. The cell free supernatant of screened isolates demonstrated antimicrobial activities against different test organisms indicating LAB isolated from Nepali fermented foods have potential alternatives as food preservative. Furthermore PCR-based detection confirmed the presence of enterocin encoding gene in 4 LAB isolates. Among 9 selected LAB isolates, all isolates were able to resist high acidic condition of pH 3 while isolates C3 (coccus) and Ca3 (rod) were able to resist pH 3 till 48 hrs of incubations. All 9 selected LAB isolates were able to resist bile salt concentration of 0.5% and 1%. Therefore, they can be able to survive in stomach high acidic and bile condition. These findings emphasize selected LAB's potential as natural antibacterial agents that could be exploited in food preservation and as probiotics to improve human gut health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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