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# Screening and Evaluation of Bacteriocin Producing *L. planatrum* and *L. pentosus* Against Food-Borne Pathogenic Bacteria Isolated from Raw Milk of Cow and Goat from Northern Region of India

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## Abstract

This study focused on inhibitory effect of *L. planatrum* and *L. pentosus* isolated from raw milk of cow and goat. The milk samples were taken because of broad spectrum against various food spoilage microorganisms. The spreading of food contaminating micro flora and food-borne pathogens bacteria is threats for food security and public health issues. In the screening of lactic acid bacteria (LAB), out of 18 strains of LAB were isolated from 56 were identified according to their morphological and biochemical characteristics. Whereas, only two isolates (Cm12 and Gm6) showed the bacteriocin producing activity. The cell-free supernatant of these two isolates are protinecous in nature having antimicrobial molecule present in the culture supernatant were found most promising to inhibit all food contaminating bacteria such as *Escherichia coli*, *Staphylococcus aureus,* and *Bacillus cereus*. Thus, further extensive research *L. pentosus* strains were considered as novel potential bacteria that were further goes for enhancing the longevity of food products.

**Keywords:** Antagonistic activity, Bacteriocin, Raw milk

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## Introduction

In general, many lactic acid bacteria (LAB) are used in the food industries as starter culture for fermentation.[1] *Lactobacilli* have been used since decades against infectious diseases and have been extensively studied for their ability to protect against pathogens. These organisms widely used as probiotics.[2] Numerous LAB are known to produce antibacterial substances including bacteriocin which can prevent the growth of pathogenic bacteria. These are natural antimicrobial peptides with bactericidal or bacteriostatic activity against some genetically closely related species. These can be classified broadly as those synthesized by gram-positive and some gram-negative bacteria. Among all synthesized by gram-positive organisms, *Lactobacilli* bacteriocins are of commercial value.

*Lactobacillus* bacteriocins are characterized as class-I bacteriocins (lantibiotics), class-II bacteriocin (heat stable, non-lantibiotics), class-III (large molecular mass and heat liable proteins with), and class IV (hydrophobic carbohydrates). Whereas, antimicrobial peptides generally disrupting the cell membrane integrity through interaction with negatively charged cell membrane by suppressing the function of DNA, RNA, or protein synthesis.[3] These bacteriocins are recognize as Generally Recognized as Safe (GRAS) by FDA[4] due to various potential characteristics rendering them useful natural food preservatives as they are small hydrophobic cationic peptides, stable at a wide range of pH, and temperature.

## Materials and Methods

Ten raw milk samples of healthy cow and goat were collecting randomly from different villages of Uttrakhand. The milk samples were collected milky from washed udder by distilled water after those samples were then collected into sterile tubes and then transferred into ice box and were taken into the laboratory within 24 h of interval.[5]

### Isolation and Phenotypic Identification of Bacteriocin Producing LAB

For makes an initial dilution ($10^{-1}$), the suspension of 10 ml milk was utilized for making serial dilution up to $10^{-6}$including 1 ml into 9 ml of sterile water. One milliliter of these dilution was poured on media (MRS) de Man Rogosa and incubating at 37°C for 48 h of incubation, different types of colonies were appear on plate with different morphological differences such as color, shape, and size and were period for catalase test as preliminary screening, the discrete colonies were picked and purified 2.3 times on fresh MRS agar media. The pure culture was further characterized by gram-staining test and cell morphology and different type of biochemical test in selection of strains.

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### Molecular Characterization of Bacteriocin Producing LAB

The molecular characterization of isolates was performed by 16s rRNA gene sequencing. In brief, DNA extracted by phenol and chloroform method.[6] Whereas, according to Marchesi *et al*., in 1998,[7] reported for the bacterial 16S rRNA and primer sequences were chosen from the conserved regions and sequencing was performed using the forward primer to (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer (5′-GGGCGGTGTGTACAAGGC-3′). PCR reactions were performed with the following conditions: Denaturation-30 cycles consisting of 95° C for 1 min, annealing-55° C for 1 min and extension-72° C for 1.5 min, followed by a final extension step of 5 min at 72° C. After cycling, the PCR products were detected by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The 16S rRNA gene sequence was analyzed by a DNA Sequencer.

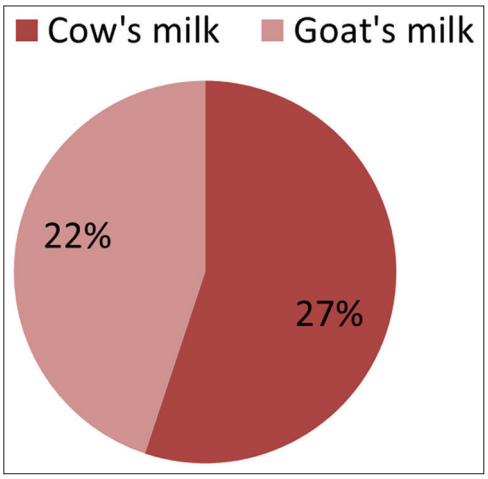


**Figure 1:** Identification of probiotic bacteria from milk samples (*n*=18)

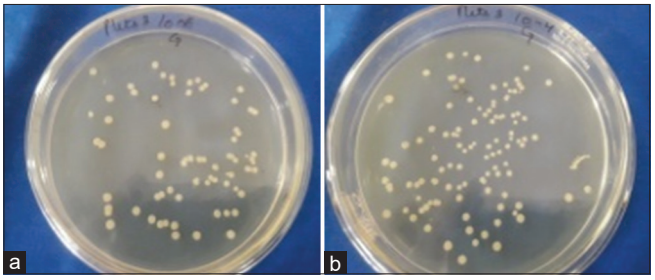


**Figure 2:** Colony morphology of (a) *L. pentosus* CM 12 and (b) *Lactobacillus planatrum* GM 6 on MRS agar

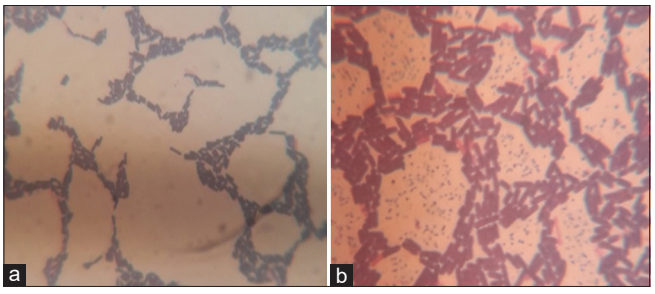


**Figure 3:** Microscopic view (1000×) of (a) *Lactobacillus planatrum* GM 6 and (b) *L. pentosus* CM 12

**Table 1:** Sampling and isolation of bacteria

| *Samples* | *Locations* | *Bacterial isolates* |
|---|---|---|
| Raw cow milk | Mangalore (Uttrakhand) | Cm1, Cm3, Cm5, Cm9, Cm12, Cm16, Cm19, Cm22, Cm27, and Cm28 |
| Raw goat milk | Mangalore (Uttrakhand) | Gm2, Gm4, Gm6, Gm10, Gm14, Gm18, Gm20, and Gm24 |

### Screening of Antibacterial Activity of Isolated LAB

The ability of LAB isolates exert an antibacterial effect against indicator microorganism (*E. coli* (ATCC 25922), *B. cerus* (ATCC 14579), and *S. aureus* (ATCC 25923) was performed by disk diffusion method. The isolated LAB strain were inoculated in 5 ml MRS broth and incubated under anaerobic condition at 30° C for 18–24 h. Cell-free supernatant (CFS) was obtained by centrifugation of this culture using centrifuge at 10.000 × g for 10 min at 4° C. To clarify the antimicrobial activity detected derived from an organic acid, hydrogen peroxide ($H_2O_2$) the CFS was adjusted to pH7.0 by adding 1N NaOH to eliminate the inhibitory effect of organic acids and catalase was added to eliminate the effect of hydrogen peroxide produce by the isolates. The disks for antimicrobial testing were prepared from Whatman filter paper and autoclaved at 121° C for 15 min. Lactic acid free MRS broth impregnated disk were used as negative control. The impregnated disks were placed on Muller–Hinton agar seeded with 18 h culture of indicator microorganism. Plates were incubated at 37° C for 24 h. Bacteriocin activities were determined by zone of inhibition around the disk.[8]

## Results and Discussion

### Isolation and Identification of Bacteriocin Producing LAB

The results focus on screening and evaluation of bacteriocin producing *L. planatrum* and *L. pentosus* against food-borne pathogenic bacteria obtaining from raw milk from northern region of Uttrakhand. In this present study, 20 raw milk samples

**Table 2:** Antimicrobial activity of bacteriocin producing isolates by disk diffusion method on Muller–Hinton agar. The data shown are average of triplicate assays with standard error

| *Isolates* | *Zone of inhibition (mm)* | | *S. aureus* |
|---|---|---|---|
| | *E. coli* | *B. cerus* | |
| Gm2 | 14.33±0.33 | 11.33±0.88 | 9.66±0.33 |
| Gm4 | 12.66±0.33 | 9.66±0.33 | 11.00±0.57 |
| Gm6 | 20.66±0.33 | 15.33±0.33 | 18±0.33 |
| Gm10 | 11.00±0.57 | - | 12.33±0.33 |
| Gm14 | 14.33±0.33 | 12.66±0.88 | 11.66±0.33 |
| Gm18 | - | 11.33±0.88 | 14.00±0.57 |
| Gm20 | 5.33±0.66 | - | - |
| Gm24 | 14.33±0.33 | 11.33±0.88 | 9.66±0.33 |
| Cm1 | 8.66±0.66 | 5.33±0.66 | 8.66±0.66 |
| Cm3 | 9.66±0.33 | - | - |
| Cm5 | 21.66±0.66 | 18.33±0.33 | 16±0.33 |
| Cm9 | 12.66±1.00 | 7.00±0.33 | 10.33±0.88 |
| Cm12 | 22.33±0.33 | 19.33±0.33 | 16.66±0.66 |
| Cm16 | - | - | - |
| Cm19 | 5.33±0.66 | 11.33±0.66 | 9.3±1.3 |
| Cm22 | 5.33±0.33 | 8.6±0.66 | 8.3±1.0 |
| Cm27 | - | - | - |
| Cm28 | 5.3±0.6 | 7.6±1.3 | 9.0±0.57 |

*Value of mean of triplicate±standard error,-No zone of inhibition

were collected from cow and goat showing in Table 1. LAB were screened and isolated raw cow milk *n* = 10/18 (27%) and raw goat milk *n* = 8/18 (22%) showing in Figure1.

## Antibacterial Activity of Bacteriocin Producing LAB

The preliminary effect shows antibacterial activity of the strain that was determined by disk diffusion method with 1M HCL/NaOH to remove the acid that could inhibit the production of pathogenic bacteria in the supernatant. Eighteen isolates presented antimicrobial effect against *S. aureus*, *B. cerus*, and *E. coli* [Table 2]. Eleven isolates show good antimicrobial activity greater than 10 mm zone, but some not might be against *S. aureus* or *B. cerus* Gm2, Gm18, Cm1, Cm5, Cm19, Cm22, and Cm28. However, two strains Gm6 and Cm12 show strong inhibition activity against three pathogens greater than 15 mm. In general, three isolates showing inhibitory effect of strains on *S. aureus*, *E. coli,* and *B. cerus* showed maximum broad spectrum and bactericidal effect, whereas Ren *et al*., 2018,[9] showing only 8 mm and 9 mm zone against pathogenic bacteria. Yi *et al*., 2016,[10] showed that *Lactobacillus* can be enhanced for inhibiting pathogenic bacteria selectively and confirmed that *L. cornyimis* XN8 exhibits a broad spectrum antimicrobial effect against *S. aureus*. The cell-free supernatant of two isolates Gm6 and Cm12 showed significant antagonist activity after treatment with sodium hydroxide and catalase for removing the effect of organic acid and $H_2O_2$. This persistence that the activity was not associated with organic acid and $H_2O_2$ but rather a substance of another nature that are protein in nature, known as bacteriocin. Our selected isolates exhibited high inhibitory activity against indicator organisms that the inhibition halo was beyond 20 mm, whereas according to Schillinger *et al*., 1989,[11] inhibition was scored positive if the width of the clear zone around the colonies of the culture strains showed antagonistic activity against pathogenic strain such as *E. coli*, *S. aureus,* and *B. cerus.*

**Table 3:** Morphological and biochemical characterization of *L. planatrum* and *L. pentosus*

| *Biochemical tests* | *Observation* |
|---|---|
| Gram's Reaction | + |
| Shape | Rod |
| Catalase | + |
| Glucose | + |
| Arabinose | + |
| Lactose | + |
| Galactose | + |
| Maltose | + |
| Ribose | + |
| Manitol | - |
| Gas formation | + |

(+) Positive test, (-) Negative test

## Morphological and Biochemical studies

Morphological and Biochemical characterization of *L. plantarum* and *L. pentosus* in which white colonies of isolates on MRS selective media showing in Figure 2, microscopic view of both rod shaped isolate showing in Figure 3. Whereas biochemical characterization showing in Table 2 in which both are gram positive rods, catalase positive, glucose, arbinose, lactose, galactose, maltose, ribose all are positive because of utilization of sugar whereas also formed gas.

## Molecular Characterization and Submission of Sequence to NCBI

Isolates Cm12 was identified by 16S rRNA sequence homology as a strain of *Lactobacillus pentosus.* The 16S rRNA sequence of the isolate Gm6 showed 99.64% identity *Lactobacillus planatrum* strain LMEM1001. The phylogenetic tree is constructed Figure 4. The 16S rRNA gene of isolate Cm12 was successfully sequenced, deposited to gene bank under accession number SUB942792. Similarly, 16SrRNA sequence of isolate Gm6 was successfully sequenced deposited to gene bank and accession number SUB9429399

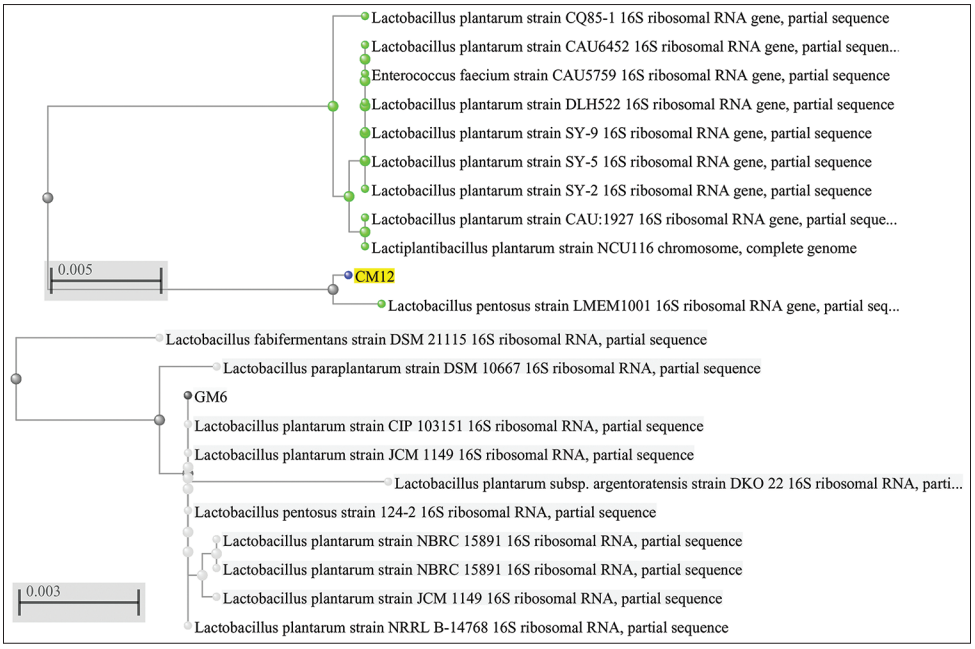


**Figure 4:** Phylogenetic tree of *L. pentosus* and *Lactobacillus planatrum*

was obtained. BLAST homology search showed 100% sequence similarity with *Lactobacillus planatrum* strain JCM 1149. As stated by Feils *et al*., 2007 that phenotypic method is an adequate method for identification of species should be confirmed by molecular method for achieve reliable methods of identification by genomic DNA of LAB.

## Conclusion

*Lactobacillus* strains isolated from raw milk of cow and goat from different localities of Uttrakhand makes them potential candidates that having properties of fight against various food-borne pathogens. *Lactobacillus* genus is the prominent flora in milk and this strain also having protein in nature called bacteriocin that has significance property to inhibitory activity. The noted fact of this work is the ability of these stain *L. planatrum* and *L. pentosus* which are novel bacteriocin suggested their remarkable application biomedical field as well as in food industries for biopreservation for preserving many food products such as dairy, meat for long time as additives instead of chemical preservation by means of their food safety as human benefits, and GRAS status in terms of their safety and GRAS status.

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