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Research Article

# Study on antibacterial activity of probiotic organism isolated from raw cow milk of Roorkee region

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**ABSTRACT**

**Lactobacillus being probiotic provides health benefits when consumed. Lactobacillus was isolated from raw cow milk at Roorkee (Uttarakhand) region. The unpasteurised raw milk was serially diluted in peptone medium and well isolated pure small colonies with entire margin were picked and preserved in MRS broth for further studies. The isolates were identified as Lactobacillus and their biochemical characteristics were determined. The isolates were positive in gram reactions, didnot possess flagella, nitrates are not reduced, gelatin is not liquefied. The probiotic nature of lactobacillus in preventing common pathogens were studied. The isolates LAB 4, LAB 15, LAB 23, LAB 42 were found to be sensitive towards *Bacillus cereus, Bacillus amyloliquifaciens, Pseudomonas auruginosa, Salmonella typhi.* The antimicrobial activity was due to their extracellular components which were proteinaceous in nature. The effective antimicrobial activity of the isolates was due to the strong acidifying property of the isolates**

**Key Words:** Lactobacillus, Cow Milk, Antimicrobial activity, Inhibitory agent, Acidification ability.

## INTRODUCTION

Probiotics were able to illustrate their constructive role towards human to modify the gutflora and replace harmful microbes[1]. During 1920's and 1930's doctors recommended the usage of probiotics for the treatment of constipation and diarrhoea which was effective for many patients. The antimicrobial compounds produced by these probiotics were administered to live stock for control of diarrhoea in humans[2]**.** Animal milk used as human food has indigenous micro flora in raw milk that plays an important role in humans and animals including the effect on the immune system[3]**.** Lactobacilli are present in milk as one of the most predominant beneficial microorganism. They are chosen to be probiotic as they improve the biological function in the host through different mechanism by sending signals to active immune cells. The deficiencies in the immune system can be repaired by stimulating the immune response because of which the host becomes resistant to infection.

Lactobacilli are a group of gram positive bacteria, non sporing, non motile cocci or rods, which produces lactic acid the major end product during the fermentation of carbohydrates. They are mostly facultative anaerobes and lack the enzyme catalase. They are strictly fermentative, aero tolerant or anaerobic, acidic/ acidophilic and have complex nutritional requirements[4] **.** They produce organic acids, hydrogen peroxide, diacetyl, inhibitor enzymes and bacteriocin[5] which exerts a strong antagonistic activity against various food contaminating organisms. Ceratin lactobacillus synthesises antimicrobial compounds that are related to bacteriocin family[6,7]. Bacteriocins are highly specific antibacterial proteins which are active against gram positive and gram negative bacteria[8]. These are potent bioactive agents wich can be used as preservatives in food industry[7].

The function of Lactobacillus as probiotics and their various involvements in development of human health aspects forms the basis for this study. The aim of the present work is to isolate and characterise lactobacillus producing bacteriocin from raw cattle milk samples from Roorkee region and to investigate the antimicrobial and antibiotic activity.

## MATERIAL AND METHODS

### a.Isolation of Lactobacillus

Raw unpasteurized milk samples of cow were collected from the local area of Roorkee, Uttrakhand during lactation period under aseptic conditions in a sterile screw cap tubes, processed within three hours and used for further studies.

Milk samples were serially diluted in peptone medium and incubated at 23°C for 30 minutes before plating by which 50% of recovery of LAB was increased. Diluted samples were plated onto **De Man Rogosa Sharpe** (MRS) medium for *Lactobacillus* isolation and incubated at 37°C for 48-72 hrs. Well-isolated colonies with typical characteristics namely pure white, small (2-3mm diameter) with entire margins were picked from each plate and transferred to MRS broth.

### b. Identification of Lactobacillus

Identification of the Lactobacilli was performed according to their morphological, cultural, physiological and biochemical characteristics[9,10]**:** Gram reaction, production of catalase, carbohydrate fermentation patterns, growth at 15°C, 30°C, 45°C and 50°C in the lactobacilli[11] broth as described by Bergy's Manual of systematic Bacteriology [9]**,** methyl red and Voges-Proskauer test in MRVP medium, nitrate reduction in nitrate broth, indole production in Tryptone broth. Purified cultures were maintained at -20°C in MRS broth with 10% glycerol and enriched in MRS broth incubated at 37°C for 24 hrs.

### c.Detection of Inhibitory activity

#### *1. Agar-Spot Test*

Lactic acid bacteria strains were cultured in 5ml of MRS broth at 30°C for 16 hrs. Aliquots (2µl) of the culture were spotted onto agar plates containing 10ml of MRS medium. After 18 hrs at 30°C, the plates were overlaid with 5ml of the appropriate soft agar (1% agar) inoculated with the cell suspension of the indicator strain *Lactobacillus acidophilus*at a final concentration of $10^5$ CFU/ml. The plates were incubated for 24-72 hrs, depending on the growth of the indicator strain, the appearances of inhibitory zones were observed. Inhibition was scored positive if the zone was wider than 2mm[12].

#### *2. Agar-Well Diffusion Assay*

The strains that were selected as potential bacteriocin producers were grown in MRS broth at 37ºC for 48 hrs. Cells were separated by centrifugation at 5000 rpm for 10 min. Around 6mm diameter wells were made on pre inoculated agar media and each well was filled with 100 µl of culture supernatant of bacteriocin producing *Lactobacillus* strains after neutralization with NaOH. Inhibitory activity was performed against certain Gram-positive and Gram-negative organisms like *Lactobacillus acidophilus* (MTCC447), *Bacillus amyloliquifaciens*(MTCC 1270), *Bacillus cereus* (MTCC 1272), *Bacillus mycoides*(MTCC 645), *Klebsiellapneumoniae*(MTCC3384), *Staphylococcus aureus* (MTCC 740), *Streptococcusfaecalis*(MTCC 459), *Pseudomonas aeruginosa* (MTCC 647), *Proteus vulgaris* (MTCC 744), and *Salmonella typhi*(MTCC 531). Inhibition zones around the wells were measured and recorded[13]**.**

#### *3. Broth Inhibitory Assay*

To test the antibacterial activity of the lactobacilli in a broth assay format, 100 µl of *Staphylococcus aureus* (MTCC1144) was added to tubes containing the culture supernatants (5ml) of the respective lactobacilli previously adjusted to pH 7.2 and supplemented up to the appropriate concentration of the test culture. Subsequently the cultures were incubated at 37˚C in aerobic conditions and after 0, 6 and 24 hrs the aliquots were collected serially and plated on Nutrient agar to determine bacterial counts[14]. Progressive reduction in the colony count at particular interval from the contact time was found.

### d.Antibiotic Susceptibility Test

Susceptibility testing was based on the agar overlay disc diffusion test[15]. LAB was grown overnight in MRS broth at 30°C under aerobic conditions. 8ml of MRS kept at 50°C were inoculated with 0.2ml of the grown culture. Petri dishes containing 15 ml of MRS were overlaid with 8.2ml of inoculated MRS and allowed to solidify at room temperature. Antibiotic discs were placed on to the overlaid plates and all plates were incubated for 20-24 hrs at 37°C under aerobic conditions. Amikacin(30µg), Ampicillin (10µg), Chloramphenicol (30µg), Gentamicin (10 µg), Erythromycin (15µg), Penicillin G (10 U), Tetracycline (30µg), Linezolid(30µg) and Vancomycin (30µg) were employed for inhibition tests. The diameter of the halos was measured[13]**.**

### e. Characterisation of the nature of inhibitory agent

The antimicrobial activity of Lactobacillus can be caused due to several factors such as acidity, hydrogen peroxide, phages and bacteriocins. Inorder to determine whether the inhibitory substances produced by bacteria were proteinaceous namely bacteriocin, sensitivity to variety of proteolytic enzymes (trypsin and alpha- chymotrypsin) was assayed[16,17]. *Staphylococcus aureus* we used as an indicator and the control was with no enzymes and inactivated enzymes[18].

**f. Acidifying ability of LAB**

Lactobacillus isolates were grown in MRS broth at 30°C for 72 hrs. The pH of the culture was determined by using pH meter to evaluate the ability of strains to acidify the culture[18].

## RESULTS AND DISCUSSION

Microorganisms were enumerated from 5 samples of raw cow milk by standard plate count technique accomplished in MRS agar media. The pure white colonies (Plate 1) with entire margins were picked up from the plates and transferred to MRS broth which was then subjected to morphological and biochemical characters are for the presence of Lactobacillus. The isolates were denoted as LAB 4, LAB 15, LAB 23, LAB 42.

### Identification of LAB

The gram positive and catalase negative strains considered as LAB[19] and were tested further. The genus Lactobacillus was classified to the species level based on morphological, physiological and biochemical characteristics (Table 1, 2). The LAB isolates were classified into the genera *Lactobacillus* based on their morphological and biochemical characters[19]. The morphological characters of all the isolates were similar. The genuses Lactobacillus vary in their shapes from long rods to short rods and also they are coccoid in shape. Lactobacillus doesn't possess flagella and don't create endospores. Nitrates are not reduced, gelatin is not liquefied, Indole is not produced and catalase negative.*L. acidophilus*,*L. salivarius*and *L. delbrueckii* subsp. *bulgaricus* isolates were specifically detected from cow milk samples. Whereas, *L. acidophilus*, *L. fermentum* and *L. pentosus* isolates were detected from buffalo milk samples. Moreover, *L. acidophilus*,*L. rhamnosus*and *L. delbrueckii* subsp. *bulgaricus* isolates were detected from ewe milk sample. However, *L. helveticus* and *L. brevis* isolate were detected from goat milk[20].

All the isolates were able to grow at 15°C. All the isolates were able to grow at 15°C. Strain LAB 4 and LAB 23 were able to grow at 30°C. Strain LAB 15 and LAB 23 were sensitive at 45°C and 50°C. LAB 42 was the only strain which was able to be sustaining at 45°C and all the four strains were sensitive to temperature 50°C. LAB isolated from rainbow trout of west Azarbaijan, Iran were Gram positive, catalase positive bacilli, were able to grow at 15°C and 45°C[21].

### Agar Spot Test

The culture supernatant obtained from the isolates was tested for antimicrobial activity against the same group of lactobacilli. All the four isolates were able to show the zone of inhibition against the indicator strain.[22] has stated that antibacterial activities were done by an agar spot in which only 14.3% of strains made known to produce bacteriocin.

### Agar Well Diffusion Assay

The positive cultures which showed zone of inhibition wider than 2mm against the indicator were tested for antibacterial activity against several gram positive and gram negative bacteria. All the four isolates were not able to inhibit *K. pnuemoniae*, *B. cereus*, *B. amyloliquifaciens*, *P. auruginosa* and *S. typhi*. LAB4 strain showed very strong inhibition against *L. acidophilus*, *S.aureus*, *P. vulgaris*. *L.acidophillus*, *Strep. faecalis* were inhibited very strongly by the isolate LAB 15. LAB 23 showed very strong inhibition against *L.acidophillus*, *Staph aureus*, *Strep. Faecalis and string inhibition against Bacillus mycoides, Klebsiella pneumonia and Proteus vulgaris*. LAB 42 showed the highest level of very strong inhibitory zone among other isolates. *L. acidophillus*, *K. pneumoniae*, *B. mycoides*, *Staph aureus*. *Strep. faecalis*,*P. vulgaris* were very strongly inhibited by LAB 42(Table 3, Plate 2). An *in vitro* study was done to determine the antibacterial activity and effective contact time of the antibacterial activity of *Lactobacillus casei* (commercial Yakult drink) against diarrheagenic organismslike *Salmonella enteritidis, Shigelladysenteriae*and *Vibrio cholerae*[23]. A new bacteriocinlactocin LC-09 produced by *Lactobacillus* strain LC-09, shows effective inhibitory activity against many species of lactobacilli and other Gram positive bacteria including *Listeria ivanovii, Streptococcus agalactii*and *Streptococcus pyogenes*[24]. Bacteriocin of *L. acidophilus* of molecular weight (M.Wt=3.5 kDa) isolated from cow had no antibacterial effect on *S. xylosus* and *Yersinia enterocolitica* and bacteriocin of *L. acidophilus* of M.Wt 6.4 kDa isolated from cow had no effect against *Yersinia enterocolitica*. While bacteriocin of *L. acidophilus* of molecular weight 4 kDa isolated from cow inhibit growth of *S. xylosus* (1.9 cm) and *Yersinia enterocolitica* (2.5 cm)[20].

### Broth inhibitory Assay

The ability of the lactobacilli to inhibit the pathogenic strain *S. aureus* in broth was also studied. The pathogen was able to grow slightly in the medium containing LAB 4, LAB 15, LAB 23 and LAB 42 after 6 hrs. After 24 hours isolates LAB 15, LAB 23, LAB 42 inhibited the growth of the pathogen.

**Table 1**
**Morphological and Physiological characteristics of the isolates**

| **Characteristics** | **Strains** | | | |
|---|---|---|---|---|
| | **LAB 4** | **LAB 15** | **LAB 23** | **LAB 42** |
| Cell Morphology | Coccobacilli | Rods | Short Rods | Cocci |
| Gram Reaction | + | + | + | + |
| Spore Formation | - | - | - | - |
| Motility | - | - | - | - |
| Catalase activity | - | - | - | - |
| Indole | - | - | - | - |
| MRVP | - | - | - | - |
| Growth at different temperature | | | | |
| 15°C | + | + | + | + |
| 30°C | + | - | + | - |
| 45°C | + | - | - | - |
| 50°C | - | - | - | - |

**Table 2**
**Biochemical characteristics of the isolates**

| **Isolate** | **Arabinose** | **Lactose** | **Mannitol** | **Melibiose** | **Salicin** | **Sorbitol** | **Sucrose** | **Raffinose** | **Trehalose** | **Glucose** | **Maltose** | **Mannose** | **Ribose** | **Xylose** | **Cellobiose** | **Probable Identity** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LAB 4 | - | + | - | - | + | + | + | - | + | + | - | + | - | + | + | *Lactococcus bacillus* |
| LAB 15 | - | + | - | + | - | - | + | + | + | + | - | + | + | + | + | *Lactobacillus fermentum* |
| LAB 23 | - | - | + | - | + | + | + | - | - | + | - | - | + | - | - | *Lactobacillus casei* |
| LAB 42 | - | - | - | - | - | - | - | - | - | + | - | | + | + | - | *Lactococcus coccus* |

The pathogen *Staphylococcus aureus* showed its pathogenecity against LAB4 even after 24 hrs (Fig 1). The similar study was carried by [23] with *Lactobacillus casei* from common yakult drink against four diarrhea causing organism where the pathogenic organism was subjected to examination for 60 minutes. The colony count decreased gradually at increase in contact time.

**Antibiotic Susceptibility Test**

The isolate LAB 42 showed its ability to resist the antibiotics at a higher range than all other isolates. LAB 42 was resistant towards all the antibiotics except for chloramphenicol and erythromycin. LAB 15 and LAB 23 strains almost showed similar range of sensitiveness and resistance towards the antibiotics. LAB 4 found to be resistant against Linezolid, Tetracycline and Vancomycin only (Table 4). Resistance to antibiotics such as chloramphenicol, ampicillin, erythromycin, tetracycline and gentamicin are generally considered transferable acquired resistances[25,26]. It is known that certain species of *Lactobacillus* are inherently resistant to ampicillin[26].

**Table 3**
**Effect of antimicrobial substances produced from the isolates on Agar platesagainst certain pathogenic organisms**

| | Bacteriocin Producing *Lactobacillus* strains | | | |
|---|---|---|---|---|
| **Indicator Strain** | **LAB 4** | **LAB 15** | **LAB 23** | **LAB 42** |
| *Lactobacillus acidophilus* (MTCC 447) | VSI | VSI | VSI | VSI |
| *Klebsiellapnuemoniae (MTCC 3384)* | NI | SI | SI | VSI |
| *Bacillus cereus* (MTCC 1272) | NI | NI | NI | NI |
| *Bacillus mycoides* (MTCC 645) | SI | SI | SI | VSI |
| *Staphylococcus aureus* (MTCC 740) | VSI | SI | VSI | VSI |
| *Streptococcus faecalis*(MTCC 459) | SI | VSI | VSI | VSI |
| *Bacillus amyloliquifaciens* (MTCC 1270) | NI | NI | NI | NI |
| *Psuedomonasauruginosa* (MTCCC 647) | NI | NI | NI | NI |
| *Salmonella typhi* (MTCC 531) | NI | NI | NI | NI |
| *Proteus vulgaris* (MTCC 744) | VSI | SI | SI | VSI |

Degree of inhibition: MI = Moderate inhibition Zone (6-9mm), SI = Strong inhibition Zone (10-14mm), VSI =very strong inhibition Zone (15-18mm), NI =No inhibition zone.

**Table 4**
**Resistant antibiotic prevalence of *Lactobacillus*isolatestoselected antibiotics by using disc diffusion test**

| S.No | Antibiotics | LAB 4 | LAB 15 | LAB 23 | LAB 42 |
|---|---|---|---|---|---|
| 1. | Chloramphenicol(30µg) | S | S | S | S |
| 2. | Ampicillin(10µg) | S | R | R | R |
| 3. | Tetracycline(30µg) | R | S | S | R |
| 4. | Amikacin(30µg) | S | S | S | R |
| 5. | Gentamicin(10µg) | S | R | R | R |
| 6. | Linezolid(30µg) | R | S | S | R |
| 7. | Erythromycin(15µg) | S | S | S | S |
| 8. | Penicillin(10u) | S | R | R | R |
| 9. | Vancomycin(30µg) | R | R | R | R |

**Table 5**
**The action of proteolytic enzymes on the crude supernatant of the isolates**

| Isolates | Trypsin | Chymotrypsin |
|---|---|---|
| LAB 4 | + | - |
| LAB 15 | - | - |
| LAB 23 | - | - |
| LAB 42 | - | - |

**Table 6**
**Acdification ability of the isolates in MRS broth at 30°C for 72 hrs**

| Isolates | Initial pH | Final pH |
|---|---|---|
| LAB 4 | 7.04 | 3.464 |
| LAB 15 | 7.05 | 3.641 |
| LAB 23 | 7.04 | 4.022 |
| LAB 42 | 7.04 | 3.671 |

Spread Plate Technique (Pure, white, small colonies with entire margins)

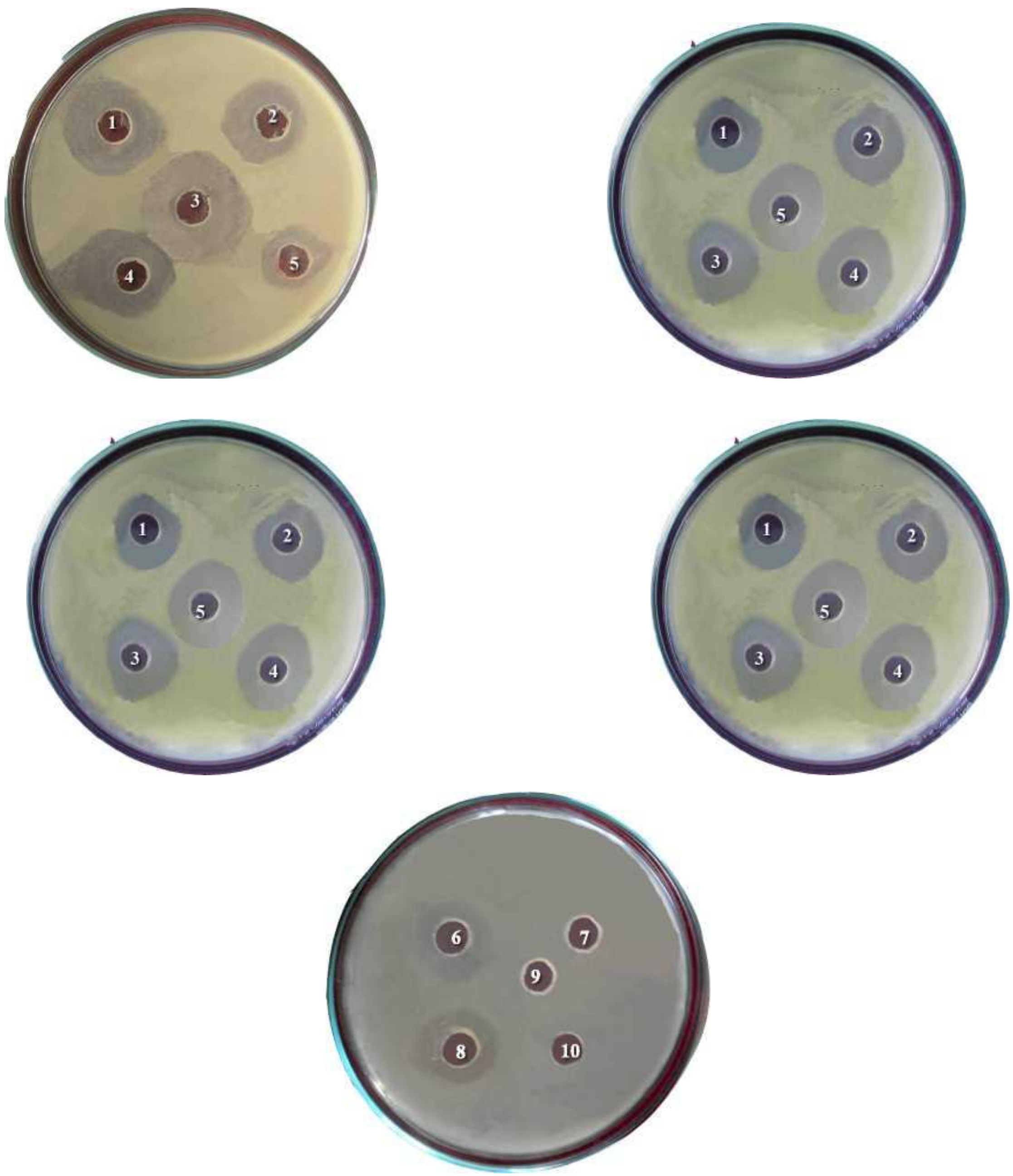
**Plate 1.**
**Morphological Identification of Lactic Acid Bacteria from different cattle samples**
***Lactobacillus* on MRS plates**

**Characterisation of the nature of the inhibitory agent**

All bacteriocins compounds are antimicrobial in nature [27,28]**.** The antibacterial substances produced by probiotic organisms are protein in nature and are secreted extracellular. The antimicrobial substance produced from strain LAB 4, LAB 15, LAB 23, LAB 42 was not able to inhibit the indicator organism because they were found to be sensitive to chymotrypsin. The action of trypsin was not able to decrease the antimicrobial activity LAB 4 (Table 5). This indicates that the active substances were secreted extracellular and was proteinaceous confirming that antimicrobial activity of the Lactobacillus was caused by bacteriocin. The action of chymotrypsin reduced totally the antimicrobial activity of Lactobacillus but trypsin enzyme cannot decrease the antimicrobial activity[29,30].

**Acidification ability of the Lactobacillus in the broth**

The isolates were incubated in MRS broth at 30° C for three days. A progressive decline in pH was observed for all strains ranging from 3.464 pH units – 2.26 pH units. The pH of LAB 23 was much decreases to 2.261 comparatively to other strains (Table 6). The strongest acidifying activity of strains confers the very effective antimicrobial activity. MRS broth when inoculated with lactobacillus showed progressive decline in pH at 30°C[31].



***1Lactobacillus acidophilus*,2 *Klebsiellapnuemoniae*,3 *Bacillus mycoides*,**
**4 *Staphylococcus aureus*,5 *Streptococcus faecalis,*6 *Bacillus amyloliquifaciens*,**
**7 *Bacillus cereus*,8 *Proteus vulgaris*,9 *Psuedomonasauruginosa*,10 *Salmonella typhi***

**Plate 2**
**Agar Well Diffussion Assay of bacteriocinproducing *LAB42*isolate**

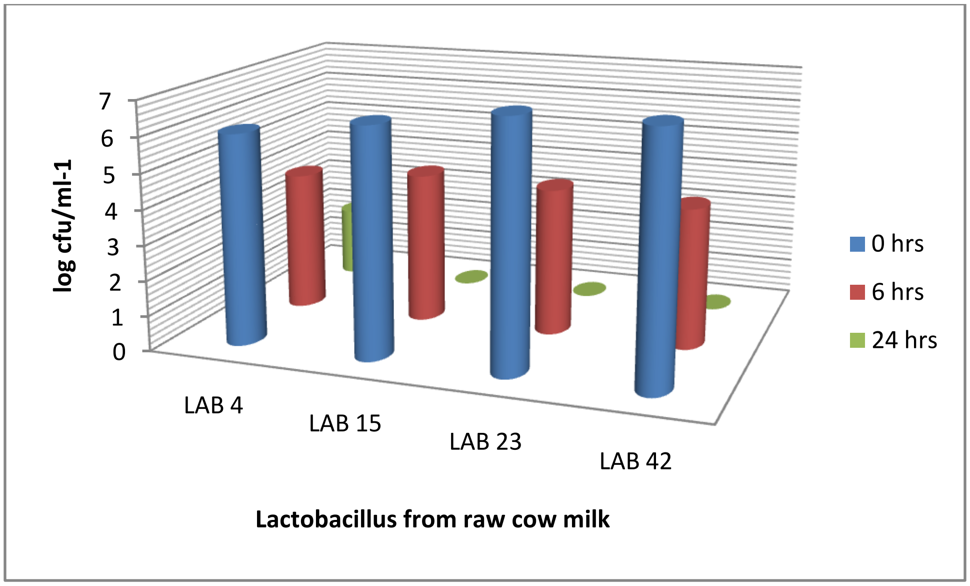



**Fig 2**
**Broth inhibitory assay of the lactobacillus isolates against *Staphylococcus aureus.***

## CONCLUSION

The probiotic organism in the milk has the remarkable efficiency in inhibiting several pathogenic microorganisms. Its antimicrobial activity acts as a barrier and develops the defense mechanism in the human system. The antimicrobial effects could be used widely in production of industrial products and its resistant nature may enable the development of probiotic therapies for several infections including cancer and can be used in development of infant probiotic products.

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