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Isolation and Characterization of Probiotic Bacteria from Various Sources

Priti Bhosale, Pooja Bhosale, Monika Sondage, Yogini Mulay and Shivaji Sathe*
Department of Microbiology, Tuljaram Chaturchand College, Baramati- 413102, Maharashtra, India

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Corresponding Author Email: sathe_sj@yahoo.com

Abstract
Probiotics are live microorganisms which when administered orally in an adequate amount improves health of humans. The aim of this study was to isolate and characterize probiotic, Lactic Acid Bacteria (LAB) from different sources like raw milk, curd, carrot and garlic. The Six isolates of (cocci and rod shape) *Lactococcus, Lactobacillus* and *Bifidobacterium* species were obtained by using selective de Man, Rogosa and Sharpe (MRS) agar medium. The isolates were identified on the basis of their cultural, microscopic and biochemical characteristics. Probiotics were live organisms which were used for the health benefits, so that the isolates were assessed to their probiotic characteristics as tolerance to pH, NaCl and Bile concentrations. In present study, results indicated that isolated LAB were able to grow in acidic pH (pH 3.5), 0-10% NaCl and tolerate 0.3% Bile concentrations. Isolated LAB has Bile Salt Hydrolase activity and also able to produce organic acid in milk. They were non-hemolytic and do not produce gelatinase. Their antibiotic sensitivity and antimicrobial activity were checked by Disc diffusion method. Results showed that isolates were sensitive to Penicillin, Ampicillin and Tetracycline and inhibit the growth of *E. coli*, *Salmonella* and *Pseudomonas*. Present study revealed that isolated species of LAB has potent Probiotic characteristics.

Keywords
Probiotics; Lactic Acid Bacteria; Raw milk; Curd; Characterization.

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INTRODUCTION:
Probiotic was a greek word meaning "for life" [1]. According to the Food and Agriculture Organization of United Nations, Probiotics was live microbial culture which when administered in an adequate amount improves health of host [2]. This concept was initially used by Nobel winner, scientist Élie Metchnikoff in 1908. He proposed its beneficial effects on health of host by using fermented products. Initially he called it as 'Bulgarian Bacillus' which was later on called as

Lactobacillus delbrueckii subsp. Probiotics improve health of host by providing vitamins, stimulating growth of good intestinal bacteria and reducing toxic activities of other harmfull bacteria. Also, they helped by reducing cholesterol level, chances of colon cancer and alimentary allergies [2,5].

Probiotic bacteria were mainly present in gastro-intestinal tract and contains more than 400 species of lactic acid bacteria (LAB). LAB was diverse group of rod or cocci shaped, Gram positive, non-sporing, non-pigmented, microaerophilic, catalase negative, nitrate positive, acid, bile and lactose tolerant bacteria [3,4]. It includes the members of different genus like *Lactococcus, Lactobacillus, Bifidobacterium* and other bacterias like *Streptococcus*, *Bacillus*, *Pediococcus*, *Leuconostoc*, some fungi and yeasts [1]. They had widespread use in food, fermentation and drug industries and Generally Recognized As Safe (GRAS) [2,4,5].

Raw milk and milk products (curd, cheese, whey), meat, wheat, rye, barley, soybean, honey, fruits and vegetables like onions, garlic were used as a rich source of probiotics [3,4]. As a probiotic, bacteria should be safe, non-toxic, non-pathogenic resistant to acids, bile and pancreatic juices and stimulate the immune system of host and that was the selection criteria for the probiotic bacteria [3,4,14]. The present study attempts to isolate and characterize such a beneficial probiotic, LAB using different sources.

MATERIALS AND METHODS:

Isolation of LAB:

In present experiment, for the isolation of lactic acid bacteria various sources like raw milk of cow and buffalo, curd, carrot, and garlic were used as samples [1,3,5]. The samples were serially diluted with sterile saline. Then 1ml from the suitable dilution was then subjected to enrichment using selective de Man's Rogosa Sharpe (MRS) medium and incubated at 37°C for 48 hours (hrs) in anaerobic and homogeneously shaken condition. MRS agar medium was composed of (gm/L) Peptone (10.0), Beef extract (10), Yeast extract (05), D-glucose (20), Tween 80 (01), K2HPO4 (02), Sodium acetate (05), Tri-ammonium citrate (02), MgSO4.7H2O (0.2) and MnSO4.4H2O (01) with pH 6.5 and agar 3% [6]. The cultures from enriched broth were then streaked on sterile plates of MRS agar medium and plates were incubated anaerobically at 37°C for 24 hrs. Selected, isolated colonies of LAB were further subcultured on sterile MRS agar plates for three times to get pure culture [1,3,5].

Identification of LAB:

Identification of isolated LAB was done on the basis of their cultural, microscopic (Gram staining and motility) and biochemical characteristics (Catalase, Oxidase, Nitrate, Gelatinase, Haemolysis and Sugar Fermentation) using Bergey's manual of determinative bacteriology [4,7,8]. The selected strains were further characterized for the various probiotic properties such as acid tolerance, bile tolerance, hemolytic activity, bile salt hydrolysis and antimicrobial activity.

Probiotic characteristics of isolated LAB:

As a *Probiotics* were live microorganisms that are used for the health benefits, so that the isolates were assessed to check their tolerance and activity against the different parameters [2,3]. In present study, tolerance of isolates to pH, NaCl concentration and bile salt concentration were determined. Activities of the isolates were checked for gelatinase production, haemolysis, bile salt hydrolysis, antibiotic sensitivity and antimicrobial activity [1,2.3,8].

Optimum growth and pH tolerance:

For this study, 2.5 to 9.5 pH ranges were selected. 1% inoculums of 24hrs old cultures of LAB were inoculated in sterile MRS broth tubes with varying pH and incubated at 37^{0}C for 24 hrs. This pH was adjusted using 0.1N HCl and NaOH. The growth was determined by measuring optical density at 560 nm [2,3,8]

Assay for NaCl tolerance:

For this study, 1 to 10% NaCl concentrations were used. 1% 24 hrs old inoculum of LAB were inoculated in sterile MRS broth tubes containing different concentrations of NaCl and that were incubated anaerobically at 37^{0}C for 24 hrs. The growth was determined by measuring optical density at 600 nm [2,3,8].

Assay for Bile tolerance:

For this study 0.1 to 0.5 concentrations of bile salt were used. 1% 24hr old inoculums of LAB were inoculated in sterile MRS broth tubes containing different bile salt concentrations. Tubes were incubated under anaerobic conditions at 37^{0}C for 24 hrs. The growth was determined by measuring optical density at 560 nm [3,8,9].

Bile salt hydrolysis activity:

Cultures of Isolated LAB were spot inoculated on MRS agar plate containing 0.3% and 0.5% bile salt which was sodium salt of Taurodeoxycholic acid (TDCA) and 0.37gm/L $CaCl_2$ [1,2,10]. Plates were incubated anaerobically at 37°C for 24 hrs. Precipitation zone around the growth of colony indicates bile salt hydrolysis (BSH) [2,10].

Quantification of organic acid:

1% of 24 hrs old culture of LAB was inoculated into MRS broth containing 10% milk sample (pH 6.6) and incubated under anaerobic condition at 37°C for 72 hrs. After every 24hrs of incubation, sample was filtered. Then by checking pH, filtrate was titrated against 0.1N NaOH using phenolphthalein as pH indicator and quantified the content of organic acid [2,3,14].

Haemolysis activity: -

24 hrs old culture of isolated LAB was spot inoculated onto sterile blood agar. plates and plates were incubated under anaerobic condition at 37ºc for 24 hrs. Plates were examined for the haemolysis activity indicated by clear zones surrounding colonies [11,12].

Assay for sensitivity to Antibiotic sensitivity:

Stock solulion of different antibiotics (Penicillin, Ampicillin, Streptomycin, Tetracycline and Erythromycin) were diluted to 0.25µg/ml. 0.1ml of 24 hrs old suspension of LAB was spreaded on sterile plates of MRS agar medium [13]. Sterile filter paper discs were deeped in antibiotic solution & placed on the above MRS agar plate. Plates were incubated anaerobically at 37ºC for 24 hrs. Observe and record the zone of inhibition [2,3,8].

Assay for Antimicrobial activity:

The antibacterial activity of isolated LAB was determined against clinically important pathogens. For this study, *E. coli, Salmonella, Pseudomonas* and *Serretia* spp. were used. 0.1 ml of suspension of these pathogens was spread on the sterile plates of nutrient agar medium [10]. 1% inoculums of LAB were added into MRS broth and incubated at 37ºC for 24 hrs. The broth was then centrifuged at 5000 rpm for 10 min and supernatant was used for antimicrobial assay using disc diffusion method. Sterile filter paper disc dipped in supernatant of LAB and were placed on above plates. Plates were incubated at 37°C for 24 hrs. Zone of inhibition was observed and recorded [1,14]. On the basis of the diameter of zone of inhibition, activities were divided into sensitive (diameter ≥ 20 mm), intermediate (20 mm ≤ diameter ≥ 10 mm) and resistant (diameter ≤ 10 mm) [15].

RESULT:

Isolation of Lactic acid bacteria:

A total six LAB were isolated from the different samples like raw milk of cow and buffalo, curd, garlic and carrot by using selective MRS agar medium [1,3]. Total isolates out of which four were cocci and two were rod shaped. They were named as Isolate1, Isolate2 up to Isolate6.

Identification of LAB:

These isolates were identified as *Lactococcus* spp, *Lactobacillus* spp and *Bifidobacterium* sppby observing their cultural, microscopic and biochemical characteristics [4,7] and which were shown in table no 1.

Isolates obtained from the raw milk (cow and buffalo), garlic and carrot were identified as *Lactococcus* spp (Isolate 1-4) and the isolates obtained from curd sample were identified as *Lactobacillus* spp (isolate 5) and *Bifidobacterium* spp (Isolate 6) [7].

Isolated LAB was cocci (*Lactococcus*) or rod (*Lactobacillus* and *Bifidobacterium*) shaped and all were Gram positive, non-motile, catalase negative, oxydase negative, nitrate positive, gelatinase negative, haemolysis negative and lactose fermenting [3,4,7].

Table no 1: Morphological and biochemical characteristics of isolated LAB

Sample used	**Cow milk**	**Buffalo milk**	**Garlic**	**Carrot**	**Curd**	**Curd**
Characteristic	**Isolate-1**	**Isolate-2**	**Isolate-3**	**Isolate-4**	**Isolate-5**	**Isolate-6**
Size	0.1mm	0.1mm	0.1mm	0.1mm	0.1mm	0.2mm
Shape	Small,circular	Circular	Small,Circular	Circular	Small,Circular	Large,Circular
Gram nature	Gram positive, Cocci	Gram positive Cocci	Gram Positive Cocci	Gram positive Cocci	Gram positive Rods	Gram positive Larger Rod
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
Catalase	Negative	Negative	Negative	Negative	Negative	Negative
Oxidase	Negative	Negative	Negative	Negative	Negative	Negative
Nitrate	Positive	Positive	Positive	Positive	Positive	Positive
Gelatinase	Negative	Negative	Negative	Negative	Negative	Negative
Hemolysis	Negative	Negative	Negative	Negative	Negative	Negative
Glucose	Positive	Positive	Positive	Positive	Positive	Positive
Lactose	Positive	Positive	Positive	Positive	Positive	Positive
Sucrose	Positive	Positive	Positive	Positive	Positive	Positive
Arabinose	Positive	Negative	Negative	Negative	Positive	Negative
Mannitol	Negative	Negative	Negative	negative	Positive	Negative

PROBIOTIC CHARACTERISTICS OF ISOLATES:

Determination of optimal growth and pH:

pH was an important factor for the growth of bacteria. Food remains in stomach for 3 hrs. *Probiotic* bacteria was used as a live microbial culture, it should tolerate to acidic pH of stomach environment (pH 3). All the isolates were able to grow in both acidic and alkaline pH [2,3,8]. In the experiment, result indicated that optimum growth of *Lactococcus* spp (Isolate1-4) and *Bifidobacterium* spp (Isolate 6) spp were at pH 3.5 and *Lactobacillus* spp (Isolate 5) was at pH 4.5. In present study, *Bifidobbacterium* spp (Isolate 5) shows comparatively good growth at acidic as well as alkaline pH conditions [2,3,8].

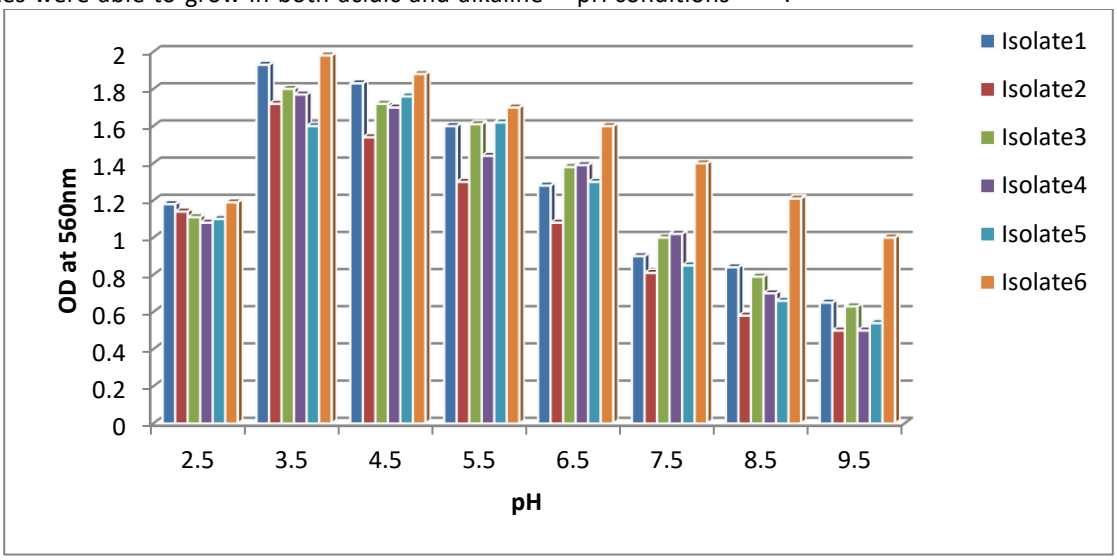


Figure 1- Effect of pH on the growth of isolated LAB

NaCl tolerance: -

NaCl act as inhibitory substance for the growth of certain types of bacteria and its tolerance was an important characteristic of probiotic bacteria. As NaCl concentration increases over 4% there was decrease in growth. Our results were matched with earlier results of Hoque et al. 2010 and Chowdhuri et al. 2012 [3,8]. All isolated LAB obtained from various sources were able to tolerate 1- 9 % of NaCl concentrations [2,3]. In this study, *Lactococcus* (Isolate 4) and *Bifidobacterium* (Isolate 6) show maximum tolerance to NaCl concentrations.

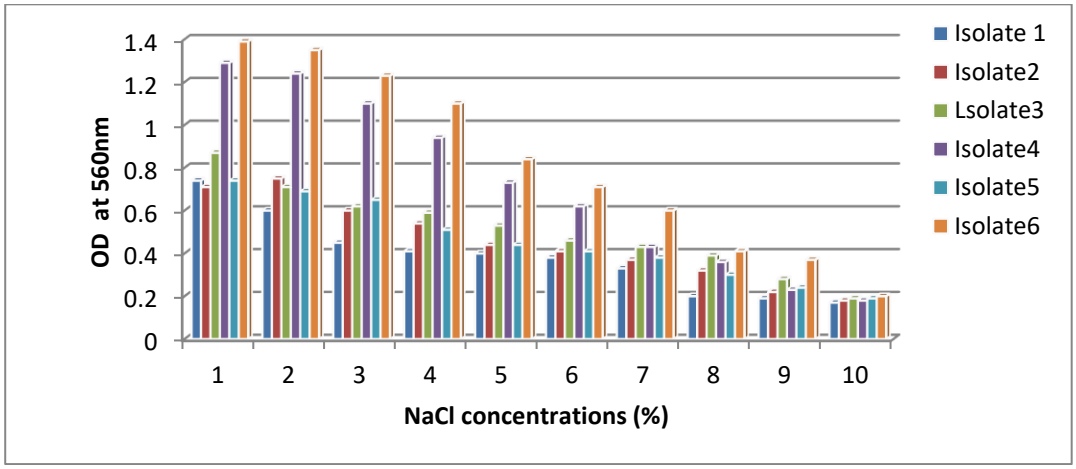


Figure 2 – Effect of NaCl concentration on the growth of isolated LAB.

Bile tolerance: -

Bile is an aqueous, yellow greenish colored solution which was synthesized in liver and secreted into duodenum (500-700ml/day) and which has antimicrobial activity. This was a selective criterion to check the in vitro survival of Probiotic bacteria in bile concentration. Intestinal bile concentration is 0.3% [3]. All the isolates of LAB were able to survive and multiply in 0.1 to 0.3% bile salt concentrations [2,3,9]. The present study revealed that *Bifidobacterium* (Isolate 6) shows comparatively good tolerance to bile concentrations (0.1 -0.5%).

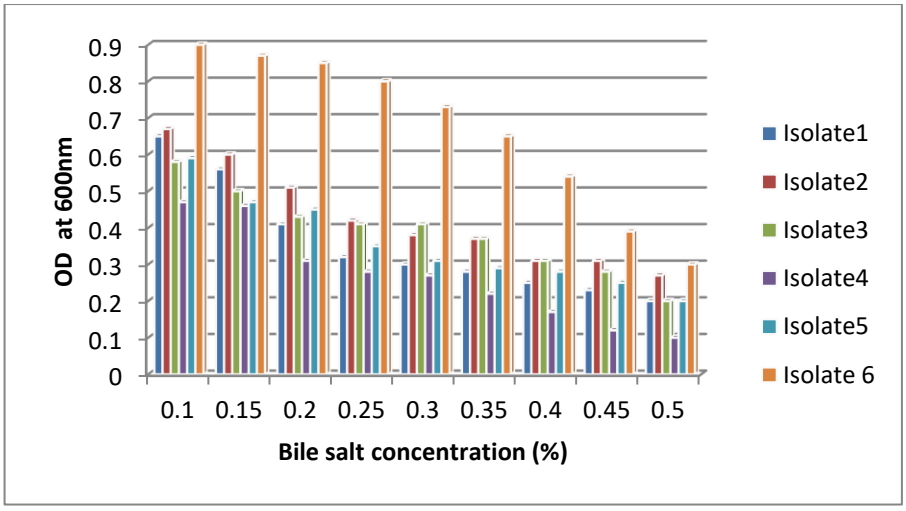


Figure 3 – Effect of bile concentrations on the growth of isolated LAB

Bile salt hydrolase (BSH) activity:
BSH was a hydrolase which catalyze the deconjugation of bile. BSH activity was also used to check in vitro survival of probiotic bacteria in presence of bile salt [1]. In present study, all the isolates showed good precipitation zone surrounding the colonies on the MRS plates supplemented with 0.3% and 0.5 % bile salt (TDCA) [2,10].

Quantification of organic acid:
The present study indicates that there was increase in organic acid production with increasing time of incubation and it causes decrease in pH of media [2,3,14]. Results were similar as earlier reported to that of Hoque et al. 2014. There were minor differences in organic acid productions by *Lactococcus*, *Lactobacillus* and *Bifidobacterium* spp in milk sample [3].
All six isolates of LAB were producing higher organic acid in milk sample after 72 hrs of incubation and at pH 2.6(Isolate 1), 2.8(Isolate 2), 2.0(Isolate 3), 2.1(Isolate 4), 1.8(Isolate 5), and 2.3(Isolate 6).

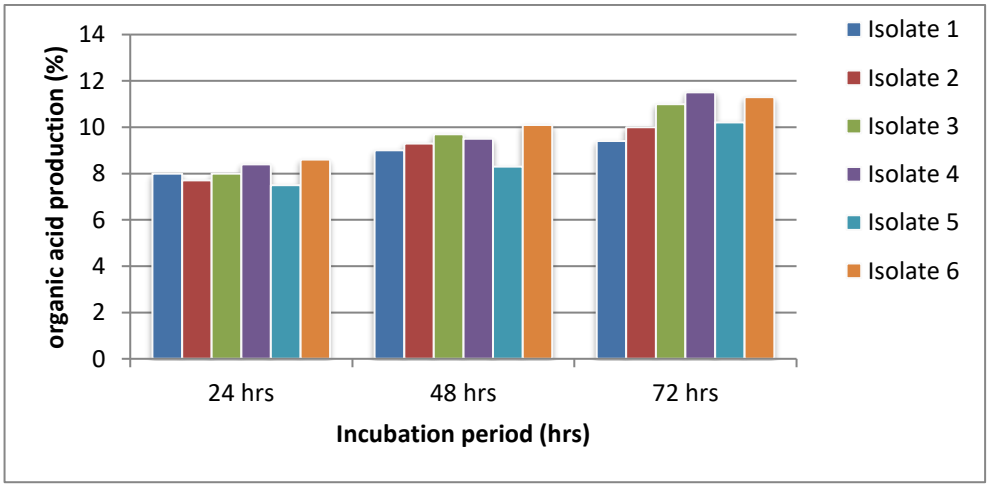


Figure 4- Organic acid production by isolated LAB

Haemolysis activity:
Haemolysis activity was an important criterion for probiotic bacteria because haemolysis would break down epithelial layers & RBCs that leads to infections [11,12]. In present study, all the isolates of LAB were haemolysis negative which were similar with the earlier results of Amina et al, 2014 [11]. The absence of hemolytic activity indicated that these bacteria were non-virulent.

Antibiotic sensitivity:
Antibiotic resistence was a new emerging concern of this era. In present study, isolated LAB were assessed to check their activity against different antibiotics using disc diffusion method [2,3,8]. The present result showed that all the isolates of LAB were sensitive to Penicillin, Ampicillin and Erythromycin while, three isolates (Isolate 1, 5 and 6) showed intermediate resistance to Streptomycin and Tetracycline which

were similar to the earlier results of Mishra et al. 2014[13].

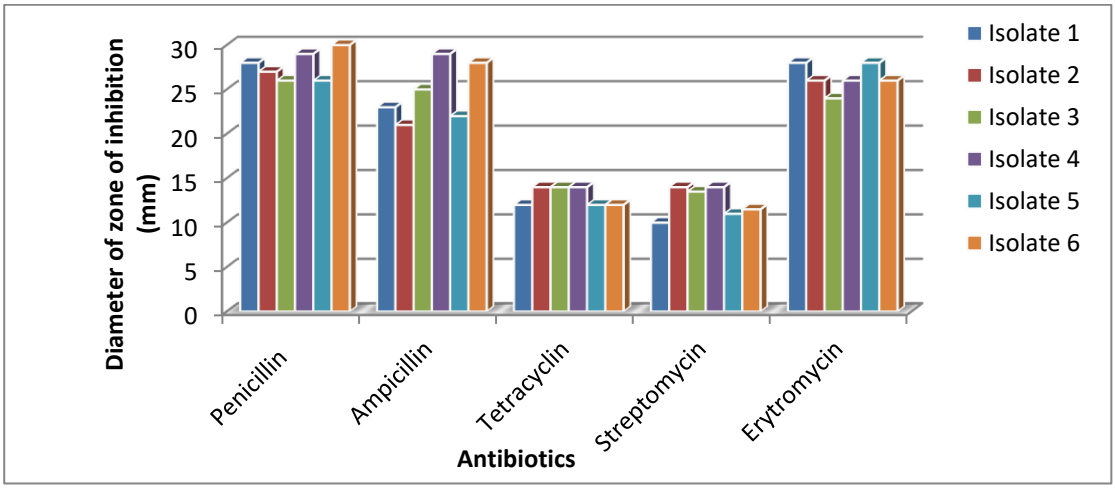


Figure 5 -Antibiotic sensitivity assay of isolated LAB

Zone of inhibition showed by *Lactococcus spp*

Antimicrobial activity:

Antibacterial activity was important criteria for the selection of Probiotic bacteria. All the isolates were assessed to check their activity against different clinically important pathogens by using disc diffusion method [1,10,14]. Isolated LAB shows good inhibition against *Pseudomonas* and *Serretia* while, intermediate to *E. coli.* This indicates the presence of bioactive molecules within LAB [15].

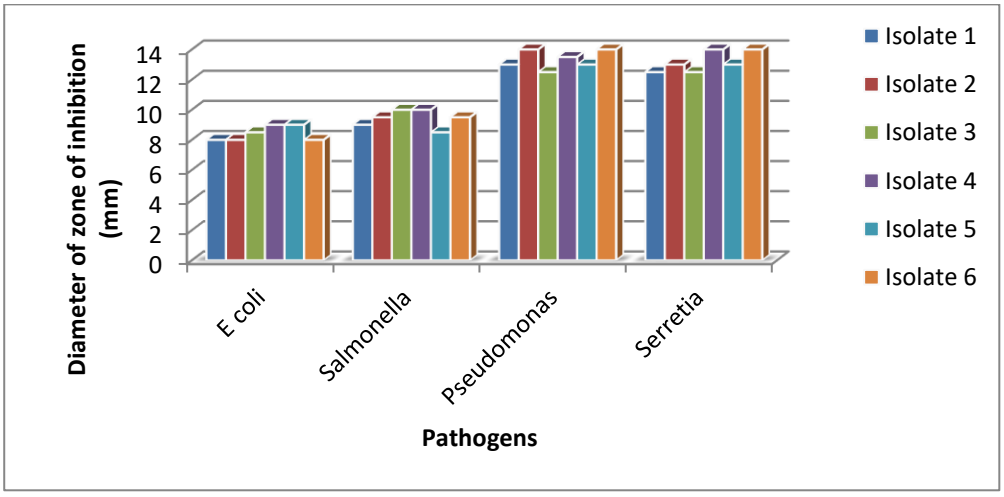


Figure 6 - Antimicrobial activity of isolated LAB

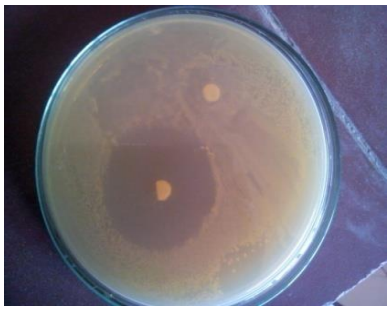
a)  b) 

Figure- 4 (a, b) Zone of inhibition shown by isolated LAB against different pathogens

CONCLUSION:

The present study showed that isolated LAB was cocci (*Lactococcus*) and rod (*Lactobacillus* and *Bifidobacterium*) shaped, Gram positive, non-motile, catalase and oxidase negative as well as nitrate positive. They were identified as species of *Lactococcus* (Isolate 1-4), *Lactobacillus* (Isolate 5) and *Bifidobacterium* (Isolate 6). These bacteria showed probiotic characteristics as they were able to tolerate acidic as well as alkaline pH, 1-10% NaCl and 0.1-0.5% Bile concentration. All isolates were non-hemolytic and able to produce organic acid in raw milk with decrease in pH. All the isolates were sensitive to Penicillin, Ampicillin and Erythromycin. Isolated LAB showed inhibitory action against different pathogens like *E. coli, Pseudomonas, Serratia* and *Salmonella*. The present study revealed that isolated *Lactococcus*, *Lactobacillus* and *Bifidobacterium* were used as potent probiotic bacteria.

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