



Isolation, Identification of Probiotic Bacteria Present in Milk

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ABSTRACT

The aim of this study was to present some data on isolation, growth, and antimicrobial activity, effect of pH, heat, and sensitivity to proteolytic enzymes of lactobacillus as probiotic bacteria. A large amount of probiotic bacteria is present in milk or milk products which are mainly lactic acid bacteria (LAB). Lactic acid bacteria (LAB) including *Lactobacillus* spp. Isolation, identification of lactic acid bacterial (LAB) was done by Gram's staining and catalase test and further confirmation was based on morphological, cultural, physiological and different biochemical tests. A total four isolates viz. *Lactobacillus fermentum*, *L. casei*, *L. acidophilus* and *bifid bacterium longum* was identified after different biochemical analysis which were also showed reliable probiotic properties. The antibacterial activity of lactic acid bacteria (LAB) isolated from raw milk against common enteric pathogens. The antagonistic properties of these isolates against *Escherichia coli*, *Staphylococcus aureus*, were examined using agar well diffusion method.

Keywords: Isolation; Probiotics; Milk; *Lactobacillus* species; Antimicrobial activities

I. INTRODUCTION

Probiotics are living, health-promoting microorganisms that are incorporated into various kinds of foods. The ability of probiotics to withstand the normal acidic conditions of the gastric juices and the bactericidal activity of the bile salts, as well as the production of lactic acid that inhibits the growth of other microorganisms, allow them to be established in the intestinal tract (Catanzaro J, 1997). The history of

probiotics began with the history of man by consuming fermented foods that is well known. (Gismondo, et al., 1999, Guarner et al., 2005) In 1908 a Russian researcher Ellie Metchnikoff, who won a Nobel Prize, firstly proposed the beneficial effects of probiotic microorganisms on human health. Metchnikoff hypothesized that Bulgarians are healthy and long lived people because of the consumption of fermented milk products which consists of rod shaped bacteria (*Lactobacillus* spp.). Therefore, these bacteria affect the gut microflora positively and decrease the microbial toxic activity. (Gismondo, et al., 1999, Çakır 2003, Chuayana, et al., 2003). Microbial cultures have been used for thousands of years in food and alcoholic fermentations, and in the past century have undergone scientific scrutiny for their ability to prevent and cure a variety of diseases. Elie Metchnikoff in 1907 first introduced the probiotics concept in 1908, when he observed the long life of Bulgarian peasants who consumed fermented milk foods. He suggested that *lactobacilli* might counteract the putrefactive effects of gastrointestinal metabolism. The concept of probiotics has evolved from the work of Metchnikoff (1908) although the term was probably first used by Lilly & Stillwell (1965).

II. MATERIALS AND METHOD:

Sample collection

Milk samples collected from local cow and buffalo were used for isolating probiotic microorganisms. The unpasteurized milk samples were added separately to MRS broth and incubated at 30°C for one week under static conditions for enrichment of microorganisms.

Isolation of lactic acid bacteria from Milk

Probiotic microorganisms were isolated by using MRS (peptone, meat extract, yeast extract, glucose, and tween (80) or MRS HIMedia. The collected samples were suspended and 0.5 ml of sample was serially diluted up to 10^{-7} concentration. About 100 μ L of each dilution were spread on MRS agar plates and then incubated at 37°C for 24 hours. The isolated colonies were sub-cultured and stored at 4°C until further use.

Identification of Lactic acid bacteria:

Identification of the selected isolates with the desired antimicrobial activity was carried out using microscopic and biochemical methods as described by **Michael et al.**, biochemical characteristics observed included Lactic acid production test, different sugar fermentation (lactose and glucose), catalase tests, pH test (growth at 6, 7, 10), Temperature test (growth at 10°C, 40°C and 60°C). Pure culture isolated on MRS agar slant was identified with the help of biochemical tests like catalase test and sugar fermentation test etc.

Microscopic identification by Gram's staining:

The colonies isolated were stained by Gram's staining method, in order to identify the morphology and gram reaction of the bacteria. A thin smear was prepared on a clean slide using the isolated individual colony. The smear was heat fixed and cooled. The dried smear was then flooded with the primary stain-crystal violet solution and allowed to stand for 1 minute. Then it was washed with water and flooded with gram's iodine solution and allowed to stand for 1 minute. The slide was then washed with decolouriser. Then the slide was flooded with a counter stain – safranin for 1 minute. After drying, the stained smear was observed under oil immersion lens 100x.

NaCl tolerance

MRS Broth with 1%, 2%, 3%, 4% NaCl (w/v) were inoculated with a 0.1 ml activated culture of bacteria isolated from stool and vaginal samples and incubated at 37°C for 48 hrs. Growth of the organism measured calorimetrically at 540 nm.

Effect of pH on growth rate:

MRS Broth with pH 3, 4, 5, 6, 7, 8 were inoculated with a 0.1 ml activated culture of bacteria incubated at 37°C for 48 hrs. Growth of the organism measured calorimetrically at 540 nm.

Effect of temperature on growth rate:

The effect of growth as well as bacteriocin production was evaluated by incubating the cultures for 24 hours at various temperatures [10°C, 40°C and 60°C].

Catalase tests:

In the center of the cavity slide the given bacterial culture was smeared. A drop of 3% H₂O₂ was added on the smear and observed for the elevation of free oxygen as bubbles which indicates positive test, whereas the negative reaction fails to form the oxygen bubbles.

Determination of Sugar Fermentation

Two different sugars; Glucose and Lactose were used for sugar fermentation assay. At first every sugar was dissolved in deionized water at a final concentration of 5% (w/v), then sterilization of sugar solutions were done by filter paper with 0.22 μ m pore diameter. MRS broth (pH 6.5) was taken into screw cap test tube and phenol red (0.01 g/L) was added into the tube as a pH indicator. Inverted Durham's tubes were placed and the medium was autoclaved at 121°C for 15 min. 1 ml different sugar solutions were inoculated into different tubes and 200 μ l overnight liquid cultures were inoculated into the broth medium. Incubation was performed anaerobically at 37°C for 24 h. Sugar fermentation was observed, as the acid production change the color of medium from its original to yellow and formation of gas in test tubes.

Inoculum preparation:

The strains of lactic acid bacteria were propagated in MRS broth at 37°C for 24 hours and bacterial biomass was collected by centrifugation at 6000rpm for 10 min, subsequently cell pellet was washed with sterile saline solution [0.85% NaCl] and was suspended in the same solution to a final optical density at 540 nm. This cell suspension was used as the inoculum for determining the growth pattern and antimicrobial activity.

Detection of antimicrobial activity:

The agar diffusion bioassay described by **Herrerros et al., (2005)** was used to screen for bacteriocin producing LAB. The liquid cultivation of the purified LAB culture was studied in MRS broth by aseptically inoculating a loop full of cells and incubated at 37°C for 24 hours under anaerobic condition. The culture broth was centrifuged at 10000 rpm for 10 min at

40°C and the inhibitory activity were determined by using the supernatant (bacteriocin like substance) of the broth after adjusting its pH to 7.0 by means of 1M sodium hydroxide to exclude antimicrobial effect of organic acids. The method adapted for the evaluation of antagonistic property was agar well diffusion assay (WDA).

Antimicrobial activity against indicator organisms:

For detection well diffusion method was used. For the agar well diffusion assay, an overnight culture of the indicator strain was used to inoculate agar growth media at 37°C. Wells of 5mm diameter were cut into N- agar plates and 10 µl of culture supernatant fluid containing antibacterial activity was added and incubated at 37°C for 24hrs and zones were observed. *Staphylococcus aureus* and *Escherichia coli* were indicator strains used against *Lactobacillus*.

III. RESULTS AND DISCUSSION

Isolation and characterization

Different Lactobacilli were isolated from the collected milk samples *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.

These isolates were given the symbol C (c₁, c₂, c₃.....and c₆) for bacterial isolates from cow milk, the symbol B (b₁, b₂, b₃.....and b₆) for bacterial isolates from buffalo milk. Description of culture morphological and microscopical characteristics was also included in Table 1.

Table1: Morphological and microscopical characteristics of LAB isolates from cow milk and buffalo milk.

Isolate sample	Colony shape	Colony size	Colony color
C1	Round, smooth,flat	2.0	White
C2	Round, smooth,flat	1.0	White
C3	Round, convex,flat	3.0	Yellowish white
C4	Round, smooth,raised	1.4	White
C5	Round, smooth,flat	3.1	White
C6	Round, smooth,flat	2.0	White
B1	Round, convex,flat	1.3	White brilliant
B2	Round, raised,flat	2.0	White
B3	Round, smooth,flat	2.0	White brilliant
B4	Round, smooth,flat	3.0	White
B5	Round, convex,flat	1.0	White
B6	Round, smooth,flat	1.5	White

Biochemical tests of isolated LAB

After confer the Lactobacillus Colonies on a MRS Himedia, we performed several biochemical tests, for the further confirmation of Lactobacillus and identification of Lactobacillus Strain. These all biochemical test shown that our isolated microorganism from Milk is may be *lactobacillus shirota* and *lactobacillus casei*. By performing Gram Staining we observed purple color gram positive rod shaped Lactobacillus species.

Table 2: Biochemical tests of isolated LAB

Isolate sample	Gram strain	Catalase test	Glucose	Lactose
C1	+ve	-ve	+ve	+ve
C2	+ve	-ve	+ve	-ve
C3	+ve	-ve	+ve	+ve
C4	-ve	-ve	-ve	+ve
C5	+ve	+ve	+ve	-ve
C6	+ve	-ve	-ve	+ve
B1	+ve	-ve	+ve	+ve
B2	-ve	+ve	+ve	+ve
B3	+ve	-ve	-ve	+ve

B4	+ve	-ve	+ve	-ve
B5	+ve	+ve	+ve	+ve
B6	+ve	-ve	+ve	+ve

Role of temperature and pH on the growth of the organism

It has been found that the growth of bacterial organism present in milk was found to be the maximum at the pH of 7.0. The optimum temperature for the maximum growth was found to be 40°C.

Table 3:

Isolate sample	Ph at 4hr	Ph at 6hr	Temp 10°c	Temp 40°c	Temp 60°c
C1	0.2±0.01	0.4±0.01	-	+++	+
C2	0.2±0.00	0.3±0.04	-	++	+
C3	0.3±0.01	0.4±0.07	-	++	+
C4	0.4±0.01	0.5±0.02	-	+++	++
C5	0.3±0.01	0.4±0.03	-	+++	+
C6	0.5±0.00	1.1±0.01	-	+++	+
B1	0.6±0.01	1.2±0.01	-	+++	+
B2	0.4±0.02	0.6±0.03	-	++	++
B3	0.5±0.07	0.8±0.01	-	+++	+
B4	0.3±0.01	0.6±0.01	-	+	+
B5	0.2±0.01	0.5±0.06	-	+++	+
B6	0.4±0.03	0.9±0.01	-	++	+

Antimicrobial activity of against test pathogens

Bacteriocin like substance was shown inhibitory effect against the growth of *Escherichia coli*



Fig 1: Antimicrobial activity of Bacteriocin like substance (different concentration) on Growth of *E.coli*.

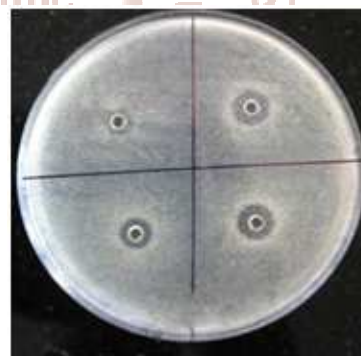


Fig 1: Antimicrobial activity of Bacteriocin Like substance (different concentration) on Growth of *Staphylococcus aureus*.

Table 4: Screening of the recovered bacterial isolates for antimicrobial activity against test pathogens.

Isolate sample	Zone of inhibition in diameter(mm)±SD	
	<i>E.coli</i>	<i>S.aureus</i>
C1	13±0.4	10±0.1
C2	11±0.0	11±0.4
C3	14±0.2	10±0.2
C4	10±0.1	0.0±0.0
C5	15±0.2	13±0.3
C6	0.0±0.0	10±0.0
B1	14±0.1	12±0.0
B2	10±0.2	9±0.2
B3	0.0±0.0	0.0±0.0

B4	13±0.1	11±0.0
B5	0.0±0.0	10±0.0
B6	12±0.3	11±0.1

IV. DISCUSSION

This study deals with the isolation, characterization and optimization of bacteriocin produced by *Lactococcus lactis* from milk. Appropriate dilutions of each sample were placed on MRS agar plates. Creamy white clones showing good colonial development was transferred to fresh MRS plates. The bacteriocins activities were examined and compared among other strains isolated. Finally, the strain showing relatively higher bacteriocins activity was selected. *Lactobacillus bulgaricus* had antibacterial activity against indicator strains *S. aureus* and *E. coli* which were further characterized. Their antimicrobial substances inactivated by trypsin and designed as bacteriocin. *Lactobacillus bulgaricus* showed the broadest range of inhibitory action. Since antibacterial activity decreased after treatment with trypsin, but not affected with lysozyme and α -amylase, so, the bacteriocins have probably a pertinacious nature. This was in accordance with **Gonzalez et al, 1994**. Also, the protein nature of bacteriocin was confirmed by its sensitivity to trypsin (**Olukoya et al, 1993**). Bacteriocin of *Lactobacillus bulgaricus* on the basis of its stability in the medium, its broad spectrum of activity on some pathogenic and spoilage food bacteria and its high potency of bacteriocin production is recommended as food preservative. In this study, production of bacteriocin was best in MRS broth, or in a medium containing peptone, yeast extract, beef extract, glucose, sodium acetate and Tween 80.

The inhibitory effect demonstrated by isolate against the indicator bacteria is an indication of possession of antibacterial activity. Results also revealed the presence of the bacteriocin in the test isolate. The production of bacteriocin the bacteria indicates its application as probiotic and as bio-preservative. Morphological and cultural studies revealed that the isolate were gram positive and gram negative and rod shaped bacteria. It was also confirmed as catalase-positive, aerobic, moderate thermophiles. The production medium should be optimized for the better production of bacteriocin. The parameters such as incubation time, pH, temperature, carbon and nitrogen sources were considered to be optimized primarily as reported by many researchers (**Trinetta et al., 2008**; **Wiese et al., 2010**). *Lactococcus lactis* was tested for

its growth under various parameters like temperature, pH, carbon and nitrogen sources. pH and temperature played an important role in cell growth and bacteriocins production. The optimum concentration of these nutrients for maximum growth as well as bacteriocin production was found to be perfect at pH 7 and 1% for glucose and bacteriological pepton respectively. All the LAB isolates survived an incubation period of 4h at pH 4.0, but exhibited variation in tolerance to acidic pH. The isolates that exhibited >80% growth when compared to controls were grouped as highly resistant (R), 70 -80% as moderately resistant (MR), 40- 60 are sensitive(S) and <40% as highly sensitive (HS). Zone of inhibition was observed against *E. coli species*. And isolate of *Staphylococcus aureus*. Antibacterial activity of the milk isolates against each test organisms was studied. *E.coli* by isolates for that table 3 shows each isolates were capable to inhibit *E.coli*. Milk isolates, C1, C4, B5 and B6 are more capable to inhibit *E.coli* as compare to others. *S.aureus* Inhibition by B1 &c4 isolates shows Maximum inhibitory zone. Isolate c3 also shows significant inhibition against *S.aureus*. Isolates have ability to inhibit *S.aureus* to treat enter toxin diseases.

V. CONCLUSION

Lactic acid bacteria were successfully isolated from milk. Probiotic activities of both the isolates were determined. From the above discussion it could be suggested that, lactic acid bacteria represent a commercial potential source of much-needed new natural antimicrobial agents, which could be promising for management of the present test pathogens.

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