



PROBIOTIC ACTIVITIES OF LACTIC ACID BACTERIA ISOLATED FROM HUMAN BREAST MILK

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ABSTRACT

Human breast milk contains potentially Probiotic lactic acid bacteria. Studies showed that this fluid has beneficial effects on the health of neonates. It is a complex species-specific biological fluid adapted to satisfy the nutritional requirements of the rapidly growing infants. These bacteria could protect the infant against infections and contribute to the maturation of the immune system. In the present studies Isolates were identified by biochemical tests and their characterization is performed by Probiotic behavior of Lactic acid bacteria.

Key words: Human breast milk, *Lactobacillus*, Probiotics, Neonates, Biological fluid, Biochemical tests

INTRODUCTION

Breast milk is regarded as the best food for rapidly-growing infants. Breast feeding protects newborn against some disease such as infections, asthma and allergy. This effect seems a result of the action of some breast milk components, like different antimicrobial compounds, immunoglobulins, immunocompetent cells and also breast milk contains Probiotic substances which stimulate the growth of the beneficial bacteria in neonate gut [1]. In a general view human milk contains fat, protein, carbohydrate, minerals and bacteria.

Additionally, it educates the infant immune system and confers a certain degree of protection against pathogens. These effects reflect the synergistic action of many bioactive molecules, present in colostrum and milk, including immunocompetent cells, [2] immunoglobulins, fatty acids, polyamines, oligosaccharides, lysozyme, lactoferrin and other glycoproteins, and antimicrobial peptides, which inactivate pathogens individually, additively, and

synergistically [3]. Breast milk is really an important factor in the initiation and development and of course composition of the neonatal gut microflora since it constitutes source of microorganisms to the infant gut for several weeks after birth [4,5]. It is estimated that an infant ingests 1×10^5 to 1×10^7 commensal bacteria while suckling if the infant consumes approximately 800 ml breast milk per day [2]. *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, or *Enterococcus faecium* were founded in breast milk and they can be regarded as potential Probiotic bacteria [6]. Hence, breast milk, a natural source of potentially Probiotic or biotherapeutic LAB [7], protects mother and infants against infectious diseases [8]. It has ability to prevent colon cancer also [9,10]. In the treatment of rotavirus diarrhoea, *Lactobacillus GG* is reported really effective [11].

MATERIAL AND METHODS

Sample collection:

Samples were voluntarily donated under aseptic conditions by 5 mothers from different locations of western Uttar Pradesh in India (district Muzaffarnagar and Meerut). Mothers declared to be in good, healthy condition, having had normal and full-term pregnancy without infant or maternal problems. The mammary areola and breast skin were carefully cleaned with soap and rinse several times with sterile water. First 500 µl of breast milk were discarded followed the release of 500-700 µl, collected in sterile carriers. Samples were immediately cooled to 5°C and stored at -20°C.

Isolation of lactic acid bacteria:

Samples were serially diluted up to 10⁻³ dilutions using sterile peptone water. 1 ml aliquots of dilutions were plated into Man, Rogosa and Sharpe agar (MRSA) (pH 6.2 and pH5.5), agar (pH 6.5) and MRS-cystein agar (pH 5.5). The plates were incubated at 37°C for 72 hrs. under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact). The media were specialized to isolate and enumerate the *lactobacilli* species. One to three bacterial colonies were randomly selected and inoculated with streak plate technique on duplicate MRSA, MRSA-cystein agar and agar plates showing growth of *lactobacilli* colonies. They were sub-cultured in MRS broth and again streaked onto MRSA to get pure colony.

Identification of the Bacterial Strains

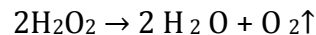
Isolates were tested for Gram-staining reaction, catalase activity and cell morphology. All Gram positive and catalase negative rods were tested for growth in MRS broth at 150C and production of gas from glucose. The tests were done according to the instructions of the manufacturer and the results were read after incubation of strains at 37°C for 3 days.

Gram's staining

Cells from fresh cultures were used for Gram staining. The Gram reaction of the isolates was determined by light microscopy. *Lactobacilli* are Gram positive. It means that they give purple-blue colour by Gram staining.

Catalase test

Catalase enzyme produced by many microorganisms that breaks down the H₂O₂ into water and oxygen that releases O₂ gas bubbles. The formation of gas bubbles indicates the presence of catalase enzyme.



Overnight cultures of isolates were grown on MRS agar at suitable conditions. After 24 hrs. 3% H₂O₂ solution was dropped onto randomly chosen colony. Fresh liquid cultures were also used for catalase activity by dropping 3% H₂O₂ solution onto 1 ml of overnight cultures. The isolates, which did not give gas bubbles, were selected as *Lactobacillus* bacteria are known as catalase negative.

Probiotic Properties of Isolates:

For the determination of Probiotic properties of isolates their resistance to low pH, tolerance against bile and salt, resistance to antibiotics and their antimicrobial activity were examined.

Resistance to Low pH

Resistance against pH 3 was observed as it resembles the pH of human stomach and it is evident that foods stays approximately for 3 hrs in stomach, as a consequence this time limit was taken into account in this study. Isolates were incubated into suitable medium (MRS) at different pH, i.e., pH 2, 2.5 and 3 for this purpose and incubated at 37°C for 48 hrs. Then 0.1 ml inoculums was transferred to MRS agar by pour plate method and incubated at 37°C for 48 hrs. The growth of LAB on MRS agar plates was used to designate isolates as acid tolerant. Viable microorganisms were enumerated at the 0, 1, 2 and 3 hrs with pour plate techniques [12, 13].

Bile Salt Tolerance

Intestinal bile concentration is believed to be 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 h. Test was applied at this concentration of bile for 4 h. MRS medium containing 0.3% bile (Oxoid) was inoculated with active cultures (incubated for 16-18h). During the incubation for 4 h, viable colonies were enumerated for every hour with pour plate technique. The growth was also monitored at OD₆₂₀.

Antimicrobial Activity

The Indicator pathogenic microorganisms were isolated from air and water on agar medium undergone overnight incubation. Active cultures were spotted on the surface of agar plates. These indicator pathogens were inoculated (1%) to soft agar containing 0.7% agar and that were overlaid on MRS plates of *Lactobacilli*. Plates were incubated to grow cultures for 24 hrs at 37°C under anaerobic conditions. Inhibition zone diameters surrounding the spotted isolates were measured (Figure 1. and Table 2.). Isolates, which gave an inhibition zone bigger than 1 mm, were determined to have antimicrobial activity [14]. All assays were performed in duplicate and the results presented were the means of duplicate trials.

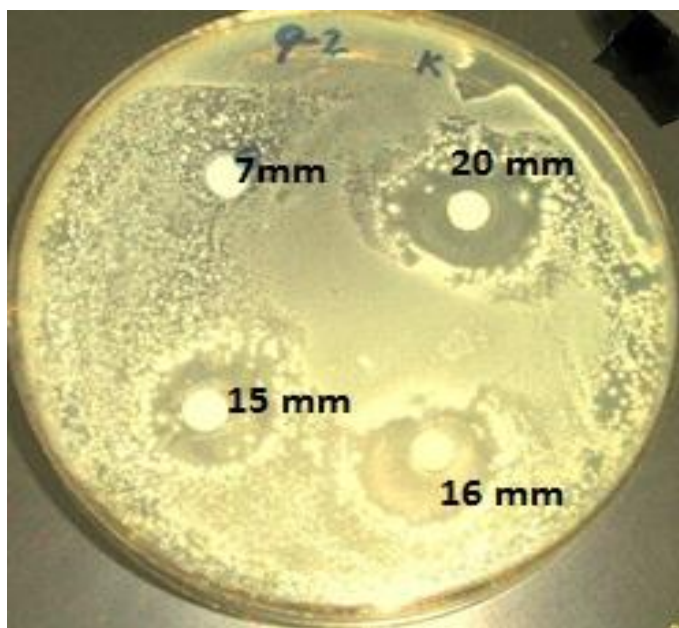


Figure 1: Culture plate showing inhibition zones created by *Lactobacillus* against various microorganisms

Antibiotic resistance

For the testing of antibiotic resistance of strain we used these antibiotics: Ampicillin, Cloramphenicol, Amoxillin. For this purpose MRS agar inoculated with bacteria, was punched and wells were made. Antibiotics were applied to wells and inhibition zones were observed to determine the antibiotic resistance of isolates.

Biochemical Characterization:

Biochemical tests were run according to methods offered by Bulut, 2003.

Gas production from glucose

MRS broths and inverted Durham tubes were prepared and inoculated with 1% overnight fresh cultures. The test tubes were incubated at 37°C for 5 days. Production of CO₂ gas in Durham tube was observed during 5 days from glucose test to determine the hetero and homo-fermentative characterization of isolates.

Growth at Different Temperatures

MRS containing Bromecresol purple indicator is prepared in 5ml tubes, used as temperature test media. The 50 µl of overnight indicator cultures were incubated for 7 days at 10°C, 15°C and 45°C. The change in colour of culture from purple to yellow was observed.

Growth at different NaCl concentrations

Tolerance of *Lactobacilli* against NaCl of different concentration was tested. 4% and 6.5% NaCl concentrations were selected. Test mediums containing Bromecresol purple indicator were prepared and transferred into 5 ml tubes. These tubes were inoculated with 1% overnight cultures and then incubated at 37°C for 7 days. The change of the color from purple to yellow was proved the cell growth.

Arginine hydrolysis test

Nessler's reagent Arginine and MRS medium were used in order to see ammonia production from arginine. MRS containing 0.3% L-arginine hydrochloride was transferred into tubes as 5 ml and inoculated with 1% overnight cultures. Tubes were incubated at 37 °C for 24 hrs. After incubation, 100 µl of cultures transferred onto a white background. The same amount of Nessler's reagent was pipetted on the cultures. The change in the color was observed. Bright orange color indicated a positive reaction while yellow indicated the negative reaction (Table 1.)

Table 1: Biochemical tests result

Strains	Properties										
	Shape	Gram's reaction	Catalase test	Glucose test	Arginine hydrolysis tets	Growth at 4% NaCl	Growth at 6.5% NaCl	Growth at 10°C	Growth at 15°C	Growth at 45°C	Antibiotic tolerance
AS1	Bacilli	+	-	+	-	+	-	-	-	+	+
AS83	Cocobacilli	+	-	+	+	+	-	-	-	+	+

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria:

Five samples of human breast milk taken from healthy mothers volunteers were used as source. Lactic Acid Bacteria were isolated at 37 °C under anaerobic conditions. Some catalase positive bacteria and yeast were also observed. The reason could be the contamination from mother's breast skin. From approximately 100 isolates, 35 isolates remained at the end of the isolation, purification and subculturing (Figure 2.). All of the isolates were gram positive catalase negative rods and cocci. It was understood that the isolates from human milk were so sensitive to the subculturing.



Figure 2. Growth on MRS Agar and MRS cysteine plates after 37 hrs incubation

Probiotic properties:

This is the main criteria of selection for Probiotic strains. Since, to reach the small intestine they have to pass through from the stressful conditions of stomach. Although in the stomach, pH can be as low as 1.0, in most in vitro assays pH 3.0 has been preferred. Due to the fact that a significant decrease

in the viability of strains is often observed at pH 2.0 and below. After the examination of all the isolates, the isolates that survive in pH 3.0 were taken to the next step. According to this experiment about 60% colonies are resist to low pH (153 out of 428). The strains which were resistant to low pH, were screened for their ability to tolerate the bile salt. The bile concentration of the human gastro intestinal tract varies but mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 hrs. [17]. Strains were detected in 0.3% during 4 hours. All of the isolates were also able to grow in 0.3% bile salt.

The selected strains were examined according to their antimicrobial activity. For this purpose, strains were detected against the indicator microorganism and they gave average 1.5 cm inhibition zone against these microorganisms (Table 2.).

Table 2. Diameters of inhibition zones created by *Lactobacillus* against various microorganisms isolated from Different sources

S. N.	Pathogen source	Diameters of inhibition zone
1.	Air	7mm
2.	Water	20 mm
3.	Soil	15mm
4.	Spoiled food	16 mm

There was no zone of inhibition on the culture plate of *lactobacillus* due to antibiotics. This proved that bacteria is resistant to used antibiotics i.e. ampicillin, amoxillin and chloramphenicol.

Biochemical characterization:

All of the isolates were subjected to Gram's staining and they were examined under light microscope. All the strains gave blue- purple colour with staining; hence they all were Gram positive bacteria. After this Gram positive isolates were

tested for catalase activity. They were all catalase negative. To test the gas production from glucose test tubes were observed for 5 days. Gas production was observed during experiment. This indicates that they were AS17 and AS83 strains [15, 16] and were heterofermentative cultures in nature. Another method for the identification the isolates was the ability of growth at different temperatures. From the results of 7 days observation, all of the isolates can grow at 45 °C however they cannot grow at 10°C and 15°C.

Growth at different NaCl concentrations was observed. All of the isolates have the ability to grow at 4% NaCl concentration but do not show the ability to grow at 6.5% NaCl concentration as they were AS17 and AS83 strains [16]. Arginine hydrolysis test was another step to follow the identification procedure. The isolates which gave the bright orange were accepted that they can produce ammonia from arginine. The yellow colour indicated negative arginine hydrolysis. When these biochemical test results are compared with the literature information (Table 1.3), it seems that AS17 is like to be *Lactobacillus oris*, AS83 is like to be *Lactobacillus fermentum*.

CONCLUSION

In conclusion, breast milk could be a good and safe source for isolation of Probiotic bacteria and to improve intestinal microflora of infants. Study will affirm their use in the development of new pharmaceutical preparation of functional foods that contain milk Probiotic for betterment of public health. The main aim of this study was to determine the Probiotic properties through different tests that were applied such as resistance to low pH and bile salt and antimicrobial activity tests etc. After the determination of potential Probiotic isolates, these isolates were characterized by phenotypic and biochemical methods. For the phenotypic characterization, morphologic examination, resistance to different temperatures and salt concentrations, gas production from glucose, ammonia production from arginine, and determination of sugar fermentation profiles were applied. The bacteria, isolated from human milk sample were found to be Probiotic as they gave all required results. Countries, where infant mortality rate is very high due to digestive disorders and food

infections, the infant Probiotic has a bright future and can help to decrease infant mortality rate. Regulation of Probiotics is still a developing field. Naturally occurring strains can be utilized with routine meal, but testing must be done before the strain can be advertised as a health supplement. Probiotics are commonly found as dairy products, but are also sold in the form of capsules and powders. Human breast milk is a relatively new source of Probiotic bacteria so the idea is to make formula more like breast milk by promoting the sorts of intestinal bacteria can be commercialize. The intake of milk microbiota may help alleviate symptoms of a many gut disorders. The genetic modification of Probiotics is a relatively new field of study that must be considered carefully. Human milk Probiotics still require much study, but the current research available indicates a promising future for the food and health industries.

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