

ORIGINAL ARTICLE

In Vitro Evaluation of Probiotic Potentials of Lactic Acid Bacteria Isolated from Human Colostrum

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ABSTRACT

The first thick milk produced immediately after the delivery is called human colostrum (HC). Its composition and functions are quite different than mature milk. It contains high levels of proteins, vitamins, immunoglobulins, carbohydrates, amino acids and many other nutrients. Apart from its nutritional aspects, HC also contains large number of Lactic Acid Bacteria (LAB) with huge probiotic potential. These LAB helps in nourishment, proper growth and development of infants in the early stages of life. The main objective of the study was to characterize and evaluate the probiotic potential of LAB from HC. The study showed several LAB with probiotic potential. The isolated LAB fulfilled all the necessary criteria of a standard probiotics such as growth at low pH, different temperatures, tolerance against bile salts, resistance against antibiotics and antimicrobial activities against common human pathogens. Four isolates of the study were found to be very promising in showing resistance against antibiotics and antimicrobial response against common pathogens such as *Escheria coli* ATCC 25922, *Proteus vulgaris* ATCC 33420, *Staphylococcus aureus* ATCC 25922, *Salmonella typhi* ATCC 733 and *Pseudomonas aeruginosa* ATCC 27853. On the basis of biochemical characterization, the isolates were identified as *Lactobacillus brevis*, *L. acetotolerans*, *L. casei* and *Pediococcus acidilactici*. The present paper deals with the isolation, characterization and evaluation of probiotic potentials of LAB isolated from HC.

Keywords : Antagonistic activity, Human Colostrum, Infant Gut, Lactic Acid Bacteria, Probiotics.

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INTRODUCTION

For many years, HC was considered to be a sterile fluid, but recent studies have revised this dogma [4]. The period of flow of HC is from 1st to 6th day of lactation. The milk produced after the 6th day is mature milk [7]. HC is a thick fluid rich in nutrients and contains vitamins, proteins, amino acids, carbohydrates and lipids along with several immune cells which provide immunity to infants in early stages of growth and development [2]. Recent studies reveals that apart from all the nutritional aspects of HC, it also contains large number of probiotic bacteria which helps in digestion and protection against infections [18]. The study on milk of Rhesus monkey (*Mucaca mulatta*) first showed that milk contains 19 different species of bacteria belonging to 8 genera in its constituents [12]. HC also contains large number of other bacteria [11]. These bacteria also play a very important role in the development of immune system of infant [27]. The number of bacteria in HC are about thrice more than mature milk. The number of bacteria in mature milk lower downs with the continuous regular flow of milk [19]. From the studies carried out in the past, majority of the bacteria isolated from human milk were generally Lactic Acid Bacteria (LAB) [13, 14]. LAB is a large group of bacteria used worldwide as a probiotic. This group of bacteria involves the microorganisms of genera *Lactobacillus*, *Lactococcus*, *Aerococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *Streptococcus*, *Sporolactobacillus*, *Vagococcus* and *Carnobacterium* [20-22]. The bacteria of these genera have high probiotic potential and have been proven safe for human consumption [8]. The first bacteria that enters the infant gut is from HC. These bacteria enters infant gut through HC and remains in the gut for entire life [21]. The gut microflora get involves in various biochemical processes and serves several functions in the welfare of human gut [6]. LAB have innumerable health benefits such as blood pressure

lowering [24], prevention of colon cancer [23], reduction of allergic symptoms [5], reduction of cholesterol [1], boosting of immune system [16], prevention of urinogenital infections [25], reduction of *Helicobacter pylori* infections [10], Intestinal Inflammation [12], antimicrobial effects on pathogens [26] and many more. Therefore, it can also be said that LAB are boon to infant gut. The present study deals with isolation, characterization and evaluation of probiotic potential of HC.

MATERIAL AND METHODS

Sample Collection:

Total 60 different HC samples were collected from lactating mothers who voluntarily gave their consent for our study. All the samples were collected immediately after the delivery from the maternity ward of Jaipur National University Hospital, Jaipur (India). The tubes used for sample collection were autoclaved using standard protocols. The nipples of lactating mothers were cleaned properly with cotton dipped in alcohol to avoid any contaminations of breast skin microflora. The mid flow of HC was carefully aseptically collected in the tubes with the help of experts.

Isolation of LAB:

The isolation of LAB from HC was quickly processed after completion of sample collection. The HC samples were serially diluted upto 10^{-6} using sterile peptone water. The last three dilutions were inoculated on MRS agar plates using Spread Plate Technique. The inoculated plates were incubated at 37°C for 48 h under anaerobic conditions using anaerobic gas jar.

Enumeration of LAB:

After incubation, the bacterial colonies were counted using digital colony counter and Colony Forming Unit (CFU) per mL of HC were calculated using standard method.

Biochemical Characterization of Isolates:

Isolated bacteria were sub-cultured to get pure form of colony. The colony characteristics of each isolate were recorded carefully. The pure colonies were further chosen for biochemical characterization. Gram's staining, Catalase test, Oxidase Test, Arginine Hydrolysis Test and Sugar Fermentation tests were performed to characterize the isolates to be LAB as per recommendation of Bergey's Manual of Determination Bacteriology.

Gram's Staining:

A single drop of sterile water was dropped on a clean glass slide and a pure colony of isolate was picked from the plate and was mixed gently to prepare a smear. The smear was heat fixed carefully. The standard procedure of Gram's staining was followed and the slide was observed under oil emulsion lens (10x X 100x) of compound microscope. As LAB are Gram positive in nature, all the isolates which showed Gram positive nature were further processed for other biochemical tests.

Catalase Test:

Catalase test was performed for all the isolates which were Gram's positive. Catalase is a type of enzyme which is produced by several microorganisms that breaks down hydrogen peroxide into water and oxygen and forms bubbles of gas. The 3% hydrogen peroxide solution was mixed gently on the surface of clean glass slide and was observed for bubble formation. As LAB are catalase negative, all the isolates that showed negative results of catalase test were further tested for oxidase test.

Oxidase Test:

All the isolates which showed catalase activity negative were further tested for oxidase test. Cytochrome c oxidase is an enzyme found in several bacterial electron transport chain. Presence of cytochrome c oxidase oxidizes the reagent called tetramethyl-phenylenediamine into indophenols (purple color) end product. As LAB are oxidase negative, all the isolates which showed negative results were further tested for its arginine hydrolysis.

Arginine Hydrolysis Test:

Nessler's reagent and arginine MRS medium were used to check the production of ammonia from arginine. 5 mL of MRS broth was transferred to empty test tube and 100 μL of test culture (O. D 1.0 at 600 nm) was inoculated and the tubes were incubated at $37\pm 1^{\circ}\text{C}$ for 24h. After incubation, an equal volume of Nessler's reagent was added to each tube. The immediate appearance of dark orange color was interpreted as positive (presence of ammonia) while indication of yellowish color was interpreted as negative reaction (absence of ammonia) [15].

Sugar Fermentation Test:

Carbohydrate when fermented by microorganisms form an acid or acid with gas at the end. Depending on the microorganisms involved, the end products may vary. All the isolates which were Gram positive and catalase and oxidase negative were tested for their sugar fermentation activity. Sugars were prepared using standard protocol (HiMedia) and each tube of sugar contained Durham's tube in inverted position.

Each isolate was inoculated in all different sugars (Glucose, Lactose, Maltose, Fructose, Mannitol, Galactose and Sucrose) to note down the breakdown of sugars into acid and/or acid + gas. Incubation for 48 h at 37°C were given to all the sugars. Results were recorded after completion of incubation period. On the basis of Sugar fermentation activity, the isolates were identified using Bergey's Manual of Systematic Bacteriology [9].

Determination of Probiotic Potential.

After biochemical characterization, all the isolates were tested for their probiotic potential by testing their growth at low pH, different temperatures, tolerance against bile salts, resistance against common human pathogens and resistance to antibiotics.

Growth at low pH:

The pH of human stomach ranges between 2 to 3. It is also believed that food eaten by us stays in stomach for at least 4 h [3]. Therefore, it is the necessary for the isolate to survive at low pH for more than 4 h. To check the growth of isolates at low pH, all the isolates were inoculated in peptone water prepared with different pH (6, 5, 4, 3, 2) for a period of 6 h. After incubation period, the isolates were inoculated on MRS agar plates and were incubated under anaerobic conditions to check their survival at different pH. All the isolates were further checked for their tolerance against bile salts.

Tolerance against Bile Salts:

The concentration of bile salts in the intestine is believed to be 0.3% (w/v) and the food eaten stays in small intestine is suggested to be 4 h [17]. Therefore, all the isolates were examined for their growth at different bile salts concentrations. Peptone water with different bile salts concentration was prepared using Oxoid and active cultures of isolates were inoculated in the medium for 6 h. After incubation, the isolates were inoculated on MRS agar plates for its viable count. The isolates which showed good growth on plates were further tested for their growth at different temperatures.

Growth at Different Temperatures:

To examine the growth of isolates at different temperature, the active cultures of isolates were inoculated on MRS agar plates and were incubated at different temperatures (25, 30, 35, 40°C) in anaerobic conditions. The isolates which showed best growth at both high as well as low temperatures were further screened for their resistance against antibiotics.

Resistance to Antibiotics:

The isolates which gave best growth at high as well as low temperature were tested for their resistance against common antibiotics using Kirby Bauer method. The isolates were spreaded on the entire surface of MH agar plates and the discs of antibiotics with different concentrations were placed on the surface of agar and gently pressed. The plates were allowed to incubate at room temperature for 24-48 h. The isolates which did not give appropriate zone of inhibition around the discs of antibiotics according to standard chart were further examined for their antimicrobial activities against common human pathogens.

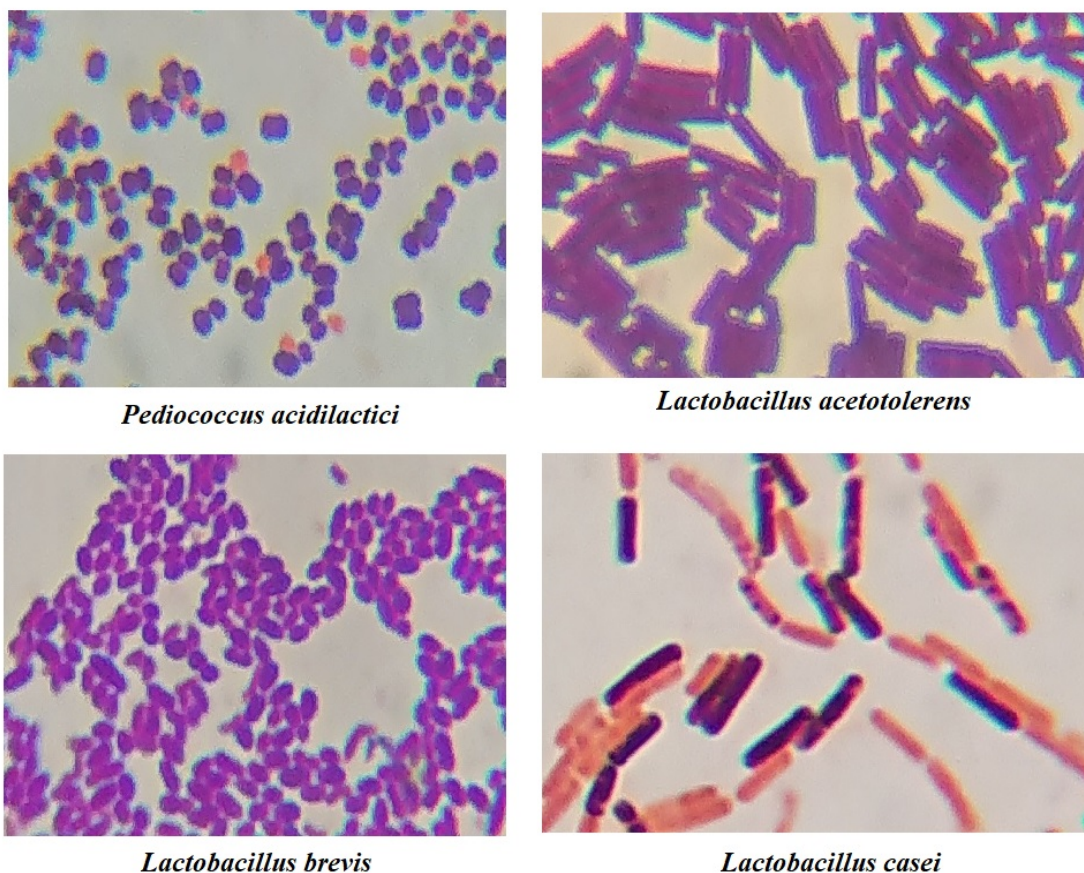
Antimicrobial activity:

All the isolates which fulfilled the above mentioned criteria were further tested for their antimicrobial activities against common human pathogens using agar well diffusion method. The indicator pathogenic microorganisms were spreaded on the entire surface of Muller Hilton (MH) agar plates and using a sterile core borer of 7 mm diameter. 5 different wells of same size were made by puncturing the MH agar plates. Using micropipettes, 80 µL of overnight grown culture of isolate were inoculated carefully in the wells. The plates were incubated for 24 h in upright position. Thereafter, the zone of inhibition were measured. The isolates which showed greater zones of inhibition were considered having good probiotic potential.

RESULTS

A total of 130 LAB were isolated from the HC of 60 different lactating mothers. The isolates were identified on the basis of physiological and biochemical characteristics. On the basis of Bergey's Manual of Systematic Bacteriology, 72 different species of LAB were identified. Of these, 4 isolates of LAB were found to be very promising with the potential of probiotics. These isolates were selected on the basis of their antimicrobial activities and their resistance against antibiotics. The average number of LAB count per ml of HC of a health lactating mothers were found to be 10^8 to 10^9 . The LAB count was measured on the basis Standard Plate Count (Total Viable Count). The isolates were initially confirmed by using biochemical test such as Catalase, Oxidase, Grams Staining, Arginine Hydrolysis test and Sugar Fermentation test. All the isolates in the present study were found Gram's positive, Catalase and Oxidase negative and also had the capacity to breakdown sugars into acids and gas [Table 1]. On the basis of their

Sugar Fermentation activity and Gram's morphology [Figure 1], the isolates were identified using Bergey's Manual of Systematic Bacteriology [9].



[Figure 1] Microscopic observation of Gram's Staining.

Determination of LAB to be potentially probiotic:

All the isolates identified as LAB through biochemical tests were further screened for determining their probiotic potential. Firstly, the growth of isolates were checked at low pH. Out of 130 isolates, 79 showed its positive growth at pH 2 which were further screened for their tolerance against different bile salts concentrations. Out of 130 total isolates, 96 were found to be prominent against tolerating the 0.3% (w/v) bile salts concentrations. These isolates were further examined for their growth at different temperatures. Out of 130 isolates, 77 showed a good growth at 40 °C and even at 25 °C. On basis of these three criteria's, 34 best isolates were selected for checking their resistance against antibiotics from which 16 best isolates were screened for testing their antimicrobial activity against common human pathogens. Out of 16 isolates, only 4 showed very high degree of zone of inhibition against pathogenic bacteria. The details of these for isolates are mentioned below in Table I, II, III and IV.

Table I: Colony characteristics of isolates with its biochemical test.

Isolate No.	Colony Characteristics							Grams Reaction	Shape	Catalase Test	Oxidase Test	Arginine Hydrolysis
	Shape	Size	Color	Opacity	Margin	Elevation	Surface					
I3201	Circular	Moderate	Creamy White	Opaque	Irregular	Raised	Dry	+	Bacilli	-	-	+
I2402	Circular	Large	Pale Yellow	Opaque	Entire	Convex	Smooth	+	Cocco-Bacilli	-	-	+
I4901	Circular	Small	Off White	Translucent	Entire	Flat	Smooth	+	Coccus	-	-	-
I5601	Circular	Medium	Pale Yellow	Opaque	Entire	Convex	Rough	+	Bacilli	-	-	+

Table II: Fermentation of different sugars for identification of isolates as per the recommendations of Bergey's Manual.

Isolate No.	Names of Sugar																Identification based on Bergey's Manual of Systematic Biology
	Glucose		Lactose		Maltose		Fructose		Xylose		Mannitol		Galactose		Sucrose		
	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	
I3201	+	-	+	-	+	-	-	-	-	-	+	+	+	-	+	+	<i>Lactobacillus casei</i>
I2402	+	-	+	-	+	+	-	-	+	+	-	-	+	+	+	+	<i>Lactobacillus brevis</i>
I4901	+	-	+	-	+	-	+	-	+	-	-	-	+	-	+	-	<i>Pediococcus acidilactici</i>
I5601	+	-	+	-	+	-	-	-	-	-	+	-	+	-	+	+	<i>Lactobacillus acetotoleren</i>

Table III: Evaluation of Resistance of isolates against common antibiotics using disc diffusion method.

Isolate No.	Names of Antibiotics													
	Erythromicin		Tetracycline		Pencillin		Gentamicin		Streptomycin		Amoxicillin		Ciprofloxacin	
Measurement on Zone of Inhibition in (mm) and its Resistance (R) or Sensitivity (S) against Antibiotics														
<i>L. casei</i>	13	R	9	R	10	R	11	R	9	R	12	R	13	R
<i>L. brevis</i>	12	R	10	R	12	R	12	R	8	R	9	R	14	R
<i>P. acidilactici</i>	9	R	11	R	8	R	9	R	10	R	10	R	10	R
<i>L. acetotoleren</i>	14	R	9	R	9	R	13	R	9	R	11	R	12	R

Table IV: Antimicrobial activity of isolated LAB against common pathogens.

Isolate No.	Names of Pathogens				
	<i>E.coli</i> ATCC-25922	<i>P. vulgaris</i> ATCC-33420	<i>S. aureus</i> ATCC -25922	<i>S. typhi</i> ATCC-733	<i>P. aeruginosa</i> ATCC-27853
<i>L. casei</i>	18 mm	17 mm	18 mm	17 mm	18 mm
<i>L. brevis</i>	21 mm	14 mm	16 mm	19 mm	20 mm
<i>P. acidilactici</i>	19 mm	18 mm	18 mm	20 mm	18 mm
<i>L. acetotoleren</i>	20 mm	16 mm	15 mm	16 mm	16 mm

DISCUSSIONS

4 best species of LAB were screened out of 130 isolates. Identification of LAB was made on the basis of colony morphology, physiological and biochemical tests as per the guidelines mentioned in Bergey's Manual of Systematic Bacteriology [9]. Similar tests performed by earlier researchers found 8 species of LAB that were Gram positive, catalase and oxidase negative and also showed active hydrolysis of arginine [16].

The acidic pH of stomach and antimicrobial actions of pepsin provide an effective barrier for LAB to survive in gastrointestinal tract [16]. For exerting beneficial effects on host, probiotic should be able to maintain its viability along the gastrointestinal transit by surviving under harsh conditions [26]. The survival rate of the isolates of our study were found to be best even at the pH of 2.

Traditional techniques of microbiology were used in the study rather than modern molecular techniques because it is more reliable. Modern techniques have some limitations such as the viability of milk microbes cannot be analyzed, total bacteria counts may be over- or underestimated because of cell-wall composition, DNA extraction methods and the number of microbial 16S gene copies which may lead to the over- or underestimation of bacteria counts. Contamination in DNA extraction kit and reagents was also reported in the past studies [19].

CONCLUSIONS

Human Colostrum contains of large number of bacteria with probiotic potential which greatly helps the infant in boosting up its immunity and in maintaining the gut microbiome. The number of LAB below this count can be a cause of worry for infant. We also found that LAB have the great potentials of fighting against common human pathogens. In our study, we have found that some LAB have great efficiency to resist against antibiotics. Such species of LAB should be commercialized and marketed at a global stage so that problems related to imbalance in gut microbiome can be solved. Through our studies, we also came to know that unnecessary consumption of antibiotics during the time of pregnancy may reduce the LAB count in HC. Therefore, use of antibiotics used be minimized. There are several other facts which are still

not known till date such as existence of LAB in HC is still a mystery. LAB in HC is a wide area of research and still needs lots of genuine studies to be carried out to solve the unknown.

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