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Isolation, Characterization and Evaluation of Probiotic Potential 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.

KEY WORDS: 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 (Fernandez et al., 2013). The period of flow of HC is from 1st to 6th day of lactation. The milk produced after the 6th day is mature milk (Castellote et al., 2011). 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 (Ballard et al., 2013). 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 (Marchesi et al., 2016). The study on milk of Rheses monkey (*Mucaca mulatta*) first showed that milk contains 19 different species of bacteria belonging to 8 genera in its constituents (Jin et al., 2011). HC also contains large number of other bacteria (Hunt et al., 2011). These bacteria also play a very important role in the development of immune system of infant (Wang et al., 2018). 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 (McGuire et al., 2015). From the studies carried out in the past, majority of the bacteria isolated from human milk were generally Lactic Acid Bacteria (LAB) (Jost et al., 2015). 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* (Pavli et al., 2018 & Neha 2019). The bacteria of these genera have high probiotic potential and have been proven safe for human consumption (Guesh et al., 2019). 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 (Pang et al., 2007). The gut microflora get involves in various biochemical processes and serves several functions in the welfare of human gut (Dunlop et al., 2015). LAB have innumerable health benefits such as blood pressure lowering (Robles-Vera et al., 2017), prevention of colon cancer (Rafter 2003), reduction of allergic symptoms (Cuello-Garcia et al., 2017), reduction of cholesterol (Agerholm-Larsen et al., 2002), boosting of immune system (King et al., 2014), prevention of urinogenital infections (Shortliffe et al., 2013), reduction of *Helicobacter pylori* infections (Hamilton 2003), Intestinal Inflammation (Jin-Sil et al., 2018), antimicrobial effects on pathogens (Tankoano et al., 2019) 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 3 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.

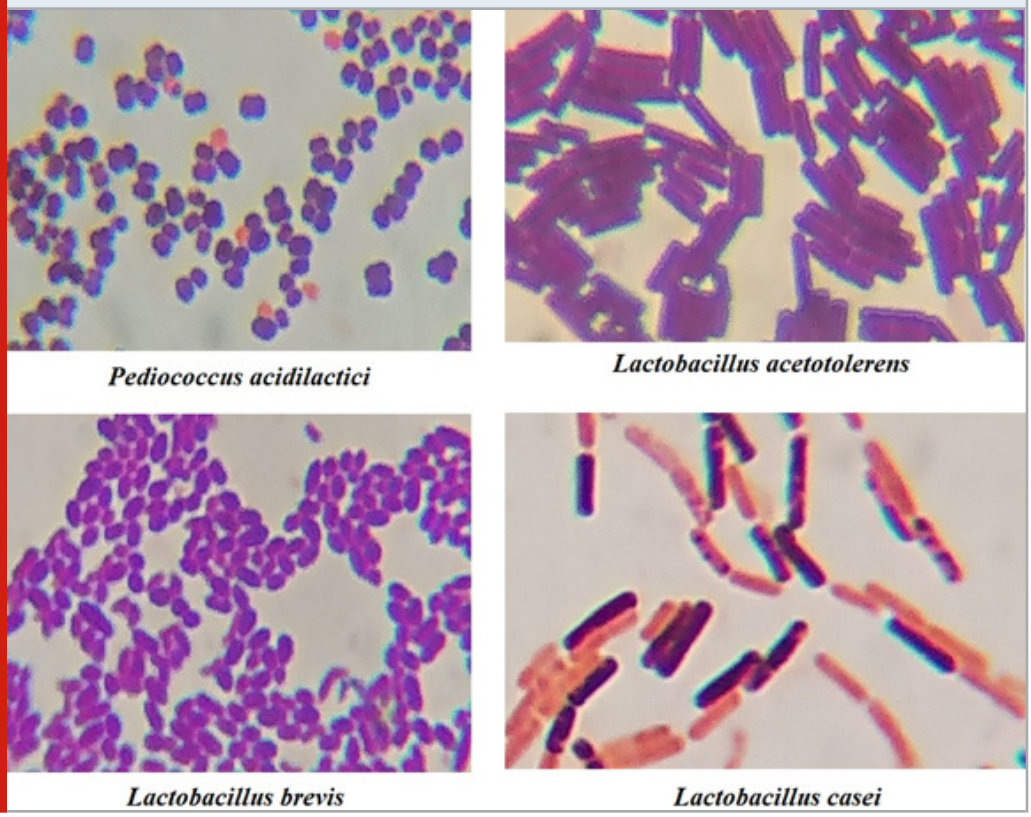
Biochemical Characterization of Isolates: Isolated bacteria were sub-cultured to get pure form of colony. The colony characteristics of each isolate were recorded carefully. The pure colonies were further chosen for biochemical characterization. Gram's staining, Catalase test, Oxidase Test, Arginine 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

Figure 1. Microscopic observation of Gram's Staining



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±1°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) (Kavitha and Devasena 2013).

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 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 (Hammes P et al., 2009). 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 (Bistha N et al., 2019). 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 (Kumari A et al., 2019). 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.

Table 1. Fermentation of different sugars for identification of isolates as per the recommendations of Bergey's Manual

Sample No.	Isolate No.	Glucose A	Glucose G	Maltose A	Maltose G	Lactose A	Lactose G	Mannitol A	Mannitol G	Galactose A	Galactose G	Fructose A	Fructose G	Sucrose A	Sucrose G	Identification Based on Bergey's Manual
HC1	I1001	+	+	+	+	+	+	-	-	+	+	+	-	+	-	*Lactobacillus acidophilus*
	I1002	+	+	+	+	+	-	-	-	+	-	+	-	+	-	*Lactobacillus sakei*
HC2	I2001	+	-	+	+	+	-	+	-	+	-	+	-	+`	-	*Lactobacillus paracasei*
	I2002	+	+	+	-	+	-	-	-	+	-	+	+	+	+	*Lactobacillus fermentum*
	I2003	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Streptococcus oralis*
HC3	I3001	+	+	+	+	+	+	-	-	+	+	+	-	+	-	*Lactobacillus gasseri*
	I3002	+	+	+	-	+	-	+	-	+	-	+	-	-	-	*Lactobacillus agilis*
HC4	I4001	+	+	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium longum*
	I4002	+	-	+	+	+	-	-	-	+	-	+	-	+`	-	*Lactobacillus rhamnosus*
HC5	I5001	+	+	+	+	+	+	+	-	+	+	+	-	+	-	*Bifidobacterium breve*
	I5002	+	+	+	+	+	-	+	-	+	+	+	+	-	-	*Pediococcus demnosus*
HC6	I6001	+	+	+	+	-	-	+	-	+	-	+	+	+	-	*Lactobacillus oris*
	I6002	+	+	+	-	+	-	+	-	-	-	-	-	+	-	*Lactobacillus curtus*
HC7	I7001	+	+	+	-	+	+	+	-	+	+	+	+	+	-	*Bifidobacterium magnum*
	I7002	+	+	+	+	+	-	-	-	+	+	+	+	+	-	*Lactococcus garvieae*
HC8	I8001	+	+	+	+	-	-	+	+	+	+	+	-	-	-	*Bifidobacterium dentium*
	I8002	+	+	+	+	+	+	-	-	+	+	-	-	+	-	*Lactobacillus johnsonii*
HC9	I9001	+	+	+	+	+	+	+	-	-	-	+	-	+	+	*Lactobacillus gasseri*
	I9002	-	-	+	+	+	-	+	-	+	-	-	-	-	-	*Lactobacillus helviticus*
	I9003	+	-	+	+	+	-	+	-	+	-	+	-	+`	-	*Lactobacillus backii*
HC10	I0101	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactobacillus parakefiri*
	I0102	+	-	+	-	+	+	-	-	+	-	+	-	+	-	*Stretococcus bovis*
HC11	I1101	+	+	+	+	-	-	+	-	+	-	+	-	-	-	*Lactobacillus silage*
	I1102	+	+	+	+	+	-	+	-	+	-	-	-	+	-	*Lactobacillus rennini*
HC12	I1201	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium bifidum*
	I1202	+	-	+	-	+	-	+	+	+	+	+	-	+	-	*Lactobacillus rapi*
HC13	I1301	+	+	+	+	+	+	+	+	+	+	+	+	+	-	*Pediococcus cellicola*
	I1302	+	+	+	+	+	-	-	-	-	-	+	-	-	-	*Lactobacillus ozensis*
HC14	I1401	+	+	-	-	-	-	+	-	+	-	+	-	+	+	*Lactobacillus helviticus*
	I1402	+	-	+	+	+	+	-	-	+	-	+	-	-	-	*Bifidobacterium hapal*
HC15	I1501	-	-	+	+	+	-	+	-	-	-	+	-	+	-	*Bifidobacterium merycicum*
	I1502	+	+	+	+	+	+	-	-	+	+	+	-	+	-	*Lactobacillus acidipiscis*
	I1503	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactococcus piscium*
HC16	I1601	+	+	+	+	+	+	+	+	+	-	+	+	+	-	*Lactobacillus acetotolerens*
	I1602	+	+	+	+	+	+	+	-	+	-	-	-		+	*Lactobacillus florum*
HC17	I1701	+	+	+	+	+	+	+	-	+	+	+	-	+	-	*Bifidobacterium breve*
	I1702	+	+	+	+	+	-	+	-	+	-	+	+	-	-	*Lactococcus plantarum*
HC18	I1801	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium reuteri*
	I1802	+	+	+	-	+	+	+	-	+	-	+	+	-	-	*Bifidobacterium bifidum*
HC19	I1901	+	+	+	-	+	-	-	-	+	-	+	+	+	+	*Lactobacillus plantarum*
	I1902	+	+	+	+	+	+	+	-	+	+	+	-	+	-	*Lactobacillus acidophilus*
HC20	I0201	+	+	+	+	+	-	+	-	+	-	+	-	-	-	*Lactobacillus agalis*
	I0202	+	+	+	+	-	-	+	+	+	+	+	-	-	-	*Bifidobacterium dentium*
HC21	I2101	-	-	+	-	+	+	+	-	+	+	+	-	+	-	*Pediococcus inopinatus*
	I2102	+	+	+	-	+	+	+	+	-	-	+	-	+	-	*Lactobacillus casei*
	I2103	+	+	+	+	+	+	-	-	+	-	+	+	+	-	*Lactobacillus larvae*
HC22	I2201	+	-	+	+	-	-	+	-	+	-	-	-	+	-	*Lactobacillus nagelii*
	I2202	+	+	+	+	+	+	+	+	-	-	+	-	+	-	*Lactococcus formosensis*
HC23	I2301	+	+	+	-	+	-	+	-	-	-	-	-	+	-	*Pediococcus stilesii*
	I2302	-	-	+	+	+	-	+	-	+	-	-	-	-	-	*Lactobacillus helviticus*
HC24	I2401	+	+	+	+	+	-	-	-	+	+	+	+	+	-	*Lactobacillus brevis*

	I2402	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus paracasei*
HC25	I2501	+	+	+	+	+	+	-	-	+	+	-	-	+	-	+	-	+	-	*Pediococcus ethanoliduran*
	I2502	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus pontis*
	I2503	-	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	*Lactococcus raffinolactis*
HC26	I2601	+	+	-	-	+	+	+	-	+	+	+	+	+	-	+	-	+	-	*Lactobacillus nuruki*
	I2602	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium reuteri*
HC27	I2701	+	-	+	-	+	+	-	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium bombi*
	I2702	+	+	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	*Lactobacillus perolens*
HC28	I2801	+	+	+	+	+	-	+	-	+	-	-	-	+	-	+	-	+	-	*Pediococcus pentosaceus*
	I2802	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus raoutii*
HC29	I2901	-	-	+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	*Lactobacillus helviticus*
	I2902	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	*Lactobacillus mobilis*
HC30	I0301	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Streptococcus ferus*
	I0302	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus buchnerii*
HC31	I3101	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus parakefiri*
	I3102	-	-	+	+	+	-	+	-	-	-	+	-	+	-	+	-	+	-	*Lactococcus hircilactis*
	I3103	+	+	+	+	+	-	+	-	+	-	+	-	+	-	-	-	-	-	*Lactobacillus agalis*
HC32	I3201	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus casei*
	I3202	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium reuteri*
HC33	I3301	+	+	+	-	+	+	+	-	+	-	+	-	+	+	-	-	-	-	*Bifidobacterium bifidum*
	I3302	+	+	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Pediococcus claussenii*
HC34	I3401	+	+	+	+	+	-	+	-	+	-	+	-	+	+	-	-	-	-	*Aerococcus suis*
	I3402	+	+	+	-	-	-	+	-	+	-	+	-	+	+	+	-	+	-	*Bifidobacterium boum*
HC35	I3501	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus pasteurii*
	I3502	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus raoutii*
HC36	I3601	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	*Lactococcus laundensis*
	I3602	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus saniviri*
HC37	I3701	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium reuteri*
	I3702	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus rogosae*
HC38	I3801	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	*Bifidobacterium dentium*
	I3802	+	-	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus sunkii*
	I3803	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	-	-	-	*Streptococcus downei*
HC39	I3901	+	+	+	+	+	+	+	-	+	-	-	-		+					*Lactobacillus florum*
	I3902	+	-	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium myosotis*
HC40	I0401	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus sakei*
	I0402	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	*Pediococcus acidilactici*
HC41	I4101	+	+	+	-	+	+	+	+	-	-	+	-	+	-	+	-	+	-	*Lactobacillus casei*
	I4102	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	-	*Lactobacillus gasseri*
HC42	I4201	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	-	-	-	*Bifidobacterium bifidum*
	I4202	+	-	+	+	-	-	+	-	+	-	-	-	+	-	+	-	+	-	*Lactobacillus acetotoleren*
HC43	I4301	+	+	+	+	+	-	+	-	+	-	-	-	+	-	+	-	+	-	*Lactobacillus rennini*
	I4302	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactococcus piscium*
HC44	I4401	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
	I4402	+	+	+	-	+	+	+	+	-	-	+	-	+	-	+	-	+	-	*Lactobacillus ozensis*
HC45	I4501	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	*Pediococcus cellicola*
	I4502	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	-	*Lactobacillus acidophilus*
	I4503	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	+	+	+	*Lactobacillus fructivorans*
HC46	I4601	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	-	-	-	*Pediococcus parvulus*
	I4602	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Lactobacillus pasteurii*
HC47	I4701	-	-	+	+	+	-	+	-	-	-	+	-	+	-	+	-	+	-	*Lactococcus hircilactis*
	I4702	+	+	+	+	+	-	+	-	+	+	+	+	-	-	-	-	-	-	*Pediococcus demnosus*
HC48	I4801	+	+	+	+	-	-	+	-	+	-	+	+	+	-	+	-	+	-	*Lactobacillus oris*
	I4802	+	-	+	+	-	-	+	-	+	-	-	-	+	-	+	-	+	-	*Lactobacillus acetotoleren*
HC49	I4901	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	*Pediococcus acidilactici*
	I4902	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium reuteri*
HC50	I0501	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+	-	+	-	*Lactococcus garvieae*
	I0502	+	+	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	*Lactobacillus perolens*

HC51	I5101	+	+	+	+	+	-	+	-	+	-	*Lactobacillus pasteurii*
	I5102	+	+	+	+	+	-	+	-	+	-	*Bifidobacterium bifidum*
HC52	I5201	+	+	+	+	-	-	+	-	+	-	-	-	*Lactobacillus perolens*
	I5202	+	+	+	-	+	+	+	-	+	+	-	-	*Aerococcus sanguinicola*
	I5203	+	+	+	-	+	-	-	-	+	-	+	+	*Lactobacillus plantarum*
HC53	I5301	+	+	+	+	+	+	+	-	+	+	+	-	*Lactobacillus gasseri*
	I5302	+	+	+	+	+	-	+	-	+	-	-	-	*Lactobacillus vini*
HC54	I5401	+	+	+	+	+	+	-	-	+	-	+	+	+	-	*Lactobacillus larvae*
	I5402	+	-	+	+	+	-	+	-	+	-	+`	-	*Lactobacillus paracasei*
HC55	I5501	-	-	+	+	+	-	+	-	-	-	+	-	+	-	*Lactococcus hircilactis*
	I5502	+	+	+	+	+	+	+	-	-	-	+	-	+	+	*Lactobacillus gasseri*
HC56	I5601	+	-	+	+	-	-	+	-	+	-	-	-	+	-	*Lactobacillus acetotoleren*
	I5602	+	+	+	+	+	+	+	+	+	+	+	+	+	-	*Lactobacillus mobilis*
HC57	I5701	+	+	+	+	+	-	+	-	+	-	+	-	-	-	*Lactobacillus agalis*
	I5702	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactococcus lactis*
	I5703	+	+	+	-	+	+	+	-	+	-	+	+	-	-	*Bifidobacterium minimum*
HC58	I5801	+	+	+	+	-	-	+	-	+	-	+	+	+	-	*Lactobacillus oris*
	I5802	+	+	+	+	+	-	+	-	+	-	+	-	+	-	*Lactobacillus casei*
HC59	I5901	+	+	+	-	+	-	+	-	-	-	-	-	+	-	*Pediococcus stilesii*
	I5902	+	+	+	-	+	-	+	-	+	-	+	-	+	-	*Bifidobacterium longum*
HC60	I0601	+	+	+	+	+	+	+	+	+	+	+	+	+	-	*Lactobacillus mobilis*
	I0602	+	+	+	+	+	-	-	-	+	+	+	+	+	-	*Lactococcus garvieae*

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) 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 AND DISCUSSION

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 108 to 109. 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 (Hammes et al., 2009).

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.

Table 2. Determination of probiotic potential based on growth at low pH, bile salt tolerance and growth at variable temperatures

Sample No.	Isolate No.	Growth at different pH				Bile Salt Tolerance (%)					Growth at different Temperatures ([])			
		pH6	pH5	pH4	pH3	pH2	0.2	0.3	0.4	0.5	25	30	35	40
HC1	I1001	+	+	+	-	-	+	+	+	-	+	+	+	+
	I1002	+	+	+	+	+	+	+	+	+	-	+	+	+
HC2	I2001	+	+	+	+	+	+	-	-	-	+	+	+	+
	I2002	+	+	+	+	+	+	+	+	-	-	-	+	+
	I2003	+	+	+	-	-	+	+	+	+	+	+	+	-
HC3	I3001	+	+	+	+	+	-	-	-	-	+	+	+	+
	I3002	+	+	-	-	-	+	+	+	+	-	+	+	+
HC4	I4001	+	+	+	+	+	+	-	-	-	+	+	+	+
	I4002	+	+	+	+	-	+	+	+	+	+	+	+	-
HC5	I5001	+	+	+	+	-	+	+	+	+	+	+	+	+
	I5002	+	+	+	+	+	-	-	-	-	-	-	+	+
HC6	I6001	+	+	-	-	-	+	+	+	+	+	+	+	-
	I6002	+	+	+	+	+	+	-	-	-	+	+	+	+
HC7	I7001	+	+	+	-	-	+	+	+	+	+	+	+	+
	I7002	+	+	+	+	+	+	+	+	+	+	+	+	+
HC8	I8001	+	+	+	+	+	+	-	-	-	+	+	+	+
	I8002	+	+	+	+	+	+	+	+	+	-	+	+	+
HC9	I9001	+	+	+	+	+	+	+	+	+	+	+	+	+
	I9002	+	+	+	-	-	+	+	+	+	+	+	+	-
	I9003	+	+	+	+	-	-	-	-	-	+	+	+	+
HC10	I0101	+	+	+	+	+	+	+	+	+	-	-	+	+
	I0102	+	+	+	+	+	+	+	+	+	+	+	+	+
HC11	I1101	+	+	+	+	-	+	+	+	+	+	+	+	+
	I1102	+	+	+	+	+	+	+	-	-	-	+	+	+
HC12	I1201	+	+	+	-	-	+	+	+	+	+	+	+	+
	I1202	+	+	+	+	+	-	-	-	-	+	+	+	+
HC13	I1301	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1302	+	+	+	+	+	+	+	+	+	-	+	+	+
HC14	I1401	+	+	-	-	-	+	+	+	+	+	+	+	-
	I1402	+	+	+	+	-	-	-	-	-	+	+	+	+
HC15	I1501	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1502	+	+	+	+	-	+	+	+	+	+	+	+	-
	I1503	+	+	+	+	+	+	+	+	+	-	-	+	+
HC16	I1601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1602	+	+	+	-	-	+	-	-	-	+	+	+	+
HC17	I1701	+	+	+	+	+	+	+	+	+	+	+	+	+
	I1702	+	+	+	+	+	+	+	+	+	-	+	+	+
HC18	I1801	+	+	-	-	-	+	+	+	+	+	+	+	+
	I1802	+	+	+	+	+	-	-	-	-	+	+	+	+
HC19	I1901	+	+	+	+	-	+	+	+	+	+	+	+	+
	I1902	+	+	+	+	+	+	+	+	+	-	+	+	+
HC20	I0201	+	+	+	+	+	+	-	-	-	+	+	+	+
	I0202	+	+	+	+	-	+	+	+	+	+	+	+	-
HC21	I2101	+	+	+	+	+	-	-	-	-	+	+	+	+
	I2102	+	+	+	-	-	+	+	+	+	+	+	+	+
	I2103	+	+	+	+	+	+	+	+	+	-	-	+	+
HC22	I2201	+	+	+	+	+	+	+	+	+	+	+	+	+
	I2202	+	+	+	+	+	+	+	-	-	+	+	+	+
HC23	I2301	+	+	+	-	-	+	+	+	+	+	+	+	+
	I2302	+	+	+	+	+	+	+	+	+	+	+	+	+

HC24	I2401	+	+	+	+	+	+	+	+	+	-	-	+	+
	I2402	+	+	+	+	+	-	-	-	-	+	+	+	+
HC25	I2501	+	+	+	+	+	+	+	+	+	+	+	+	+
	I2502	+	+	+	-	-	+	+	+	+	+	+	+	-
	I2503	+	+	+	+	+	+	-	-	-	+	+	+	+
HC26	I2601	+	+	+	+	-	+	+	+	+	+	+	+	+
	I2602	+	+	+	+	+	+	+	+	+	+	+	+	+
HC27	I2701	+	+	-	-	-	+	+	+	+	-	-	+	+
	I2702	+	+	+	+	+	+	+	+	+	+	+	+	+
HC28	I2801	+	+	+	+	+	+	+	+	+	+	+	+	+
	I2802	+	+	+	-	-	+	+	+	+	-	+	+	-
HC29	I2901	+	+	+	+	+	+	+	-	-	+	+	+	+
	I2902	+	+	+	+	-	+	+	+	+	+	+	+	+
HC30	I0301	+	+	-	-	-	+	+	+	+	+	+	+	+
	I0302	+	+	+	+	-	+	+	+	+	+	+	+	-
HC31	I3101	+	+	+	+	+	-	-	-	-	+	+	+	+
	I3102	+	+	+	+	+	+	+	-	-	+	+	+	+
	I3103	+	+	+	-	-	+	+	+	+	-	-	+	+
HC32	I3201	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3202	+	+	+	+	+	+	-	-	-	+	+	+	+
HC33	I3301	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3302	+	+	+	-	-	+	+	+	+	+	+	+	-
HC34	I3401	+	+	+	+	+	-	-	-	-	+	+	+	+
	I3402	+	+	-	-	-	+	+	+	+	+	+	+	+
HC35	I3501	+	+	+	+	+	+	+	+	+	-	+	+	+
	I3502	+	+	+	+	+	+	+	+	+	+	+	+	+
HC36	I3601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3602	+	+	+	-	-	+	+	+	+	+	+	+	+
HC37	I3701	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3702	+	+	+	+	+	+	-	-	-	+	+	+	+
HC38	I3801	+	+	+	+	+	+	+	+	+	-	-	+	+
	I3802	+	+	-	-	-	+	+	+	+	+	+	+	-
	I3803	+	+	+	+	-	-	-	-	-	+	+	+	+
HC39	I3901	+	+	+	+	+	+	+	+	+	+	+	+	+
	I3902	+	+	+	+	+	+	+	-	-	+	+	+	+
HC40	I0401	+	+	+	+	-	+	+	+	+	-	-	+	+
	I0402	+	+	+	+	-	+	+	+	+	+	+	+	+
HC41	I4101	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4102	+	+	-	-	-	+	+	+	+	+	+	+	-
HC42	I4201	+	+	+	+	+	+	-	-	-	+	+	+	+
	I4202	+	+	+	+	+	+	+	+	+	+	+	+	+
HC43	I4301	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4302	+	+	+	+	-	-	-	-	-	+	+	+	+
HC44	I4401	+	+	+	+	-	+	+	+	+	-	-	+	+
	I4402	+	+	+	+	+	+	+	-	-	+	+	+	+
HC45	I4501	+	+	+	+	+	-	-	-	-	+	+	+	+
	I4502	+	+	+	-	-	+	+	+	+	+	+	+	-
	I4503	+	+	+	+	+	+	+	+	+	+	+	+	+
HC46	I4601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4602	+	+	+	+	-	-	-	-	-	+	+	+	+
HC47	I4701	+	+	+	+	+	+	+	+	+	-	-	+	+
	I4702	+	+	-	-	-	+	+	+	+	+	+	+	-
HC48	I4801	+	+	+	+	+	+	+	+	+	+	+	+	+
	I4802	+	+	+	+	+	+	+	+	+	+	+	+	+
HC49	I4901	+	+	+	+	+	-	-	-	-	+	+	+	+
	I4902	+	+	+	+	+	+	-	-	-	+	+	+	+
HC50	I0501	+	+	-	-	-	+	+	+	+	+	+	+	-

	I0502	+	+	+	+	+	+	+	+	+	+	+	+	+
HC51	I5101	+	+	+	+	+	+	+	+	+	-	-	+	+
	I5102	+	+	+	+	-	-	-	-	-	-	+	+	-
HC52	I5201	+	+	+	+	+	+	+	+	+	+	+	+	+
	I5202	+	+	+	+	+	+	+	+	+	+	+	+	+
	I5203	+	+	+	+	+	+	+	-	-	+	+	+	+
HC53	I5301	+	+	+	+	+	+	+	+	+	-	+	+	+
	I5302	+	+	+	-	-	+	+	+	+	-	+	+	-
HC54	I5401	+	+	+	+	-	+	+	+	+	+	+	+	+
	I5402	+	+	+	+	+	+	+	+	+	+	+	+	+
HC55	I5501	+	+	+	+	-	-	-	-	-	+	+	+	+
	I5502	+	+	+	+	+	+	+	+	+	+	+	+	+
HC56	I5601	+	+	+	+	+	+	+	+	+	+	+	+	+
	I5602	+	+	+	+	+	+	+	+	+	-	+	+	+
HC57	I5701	+	+	+	+	+	+	+	+	+	-	-	+	+
	I5702	+	+	+	+	-	+	+	+	+	+	+	+	+

Table 3. Evaluation of Resistance of isolates against common antibiotics using disc diffusion method

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

Table 4. Antimicrobial activity of isolated LAB against common pathogens

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

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 and even at 25 . 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.

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 (Hammes et al., 2009). 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 (Kang et al., 2019). The acidic pH of stomach and antimicrobial actions of pepsin provide an effective barrier for LAB to survive in gastrointestinal tract (Kang et al, 2019). For exerting beneficial effects on host, probiotic should be able to maintain its viability along the gastrointestinal transit by surviving under harsh conditions (Tongwa et al., 2019). 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 (Mc Guire, 2015).

CONCLUSION

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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