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ORIGINAL ARTICLE **OPEN ACCESS**

Genotypic and phenotypic characterization of *Lactococcus lactis* strain with high probiotic potentials isolated from human colostrum

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ABSTRACT

The first thick milk produced immediately after the delivery is called human colostrum. It is biochemically and functionally different than mature milk. The period of flow of human colostrum in healthy lactating mothers is from the 1st to 6th day to delivery. Human Colostrum contains large amount of minerals and nutrients in its composition. Apart from all the nutritional aspects, human colostrum also contains large amount of potentially probiotic lactic acid bacteria. These bacteria play an important role in immune system maturation of infant. Large number of infants throughout the world faces a deficiency of probiotics in their initial stages of life due to several factors. The present study was carried on Lactococcus lactis which was isolated from human colostrum. L. lactis was found to be very prominent in showing antimicrobial activity against pathogen as well as was able to resist against antibiotics. The primary objective of the current study was to study the biochemical and molecular characteristics of L. lactis with high probiotic potentials. According to results, L. lactis showed similar homology with several strains of Lactococcus which was evaluated using several tools of bioinformatics. The present study will highlight the molecular as well as biochemical characterization of L. lactis which was isolated from human colostrum.

Keywords*: Human colostrum, L.lactis, Genotypic and phenotypic characterization*

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INTRODUCTION

Human colostrum (HC) is one of the most basic primary necessity of infant to survive in initial days of life. It is very rich in carbohydrates, vitamins, immunoglobulins, proteins, lipids and several other immune factors. Since years, HC was considered to be a sterile thick fluid. But recent studies confer that HC contains large number of mutualistic commensal bacteria that acts as a probiotic in infant gut [1]. These group of bacteria are generally found to be Lactic acid bacteria (LAB). LAB is large group of bacteria that have several health benefits on humans and are used worldwide as probiotics [2]. These group of bacteria are generally found in milk products and decomposing plants which produces lactic acid as their major metabolic end product on fermenting carbohydrates [3]. Several LAB produces proteinaceous bacteriocins which becomes a hurdle for pathogenic microorganisms [4]. LAB has been evidenced by their generally recognized as safe (GRAS) status due to their contribution to healthy microbiota in human mucosal surfaces as well as their several health benefits [5]. The group of LAB comprises of several genera such as *Lactobacillus, Pediococcus, Aerococcus, Leuconostoc, Streptococcus, Lactococcus and Enterococcus* [6]. *L. lactis* belongs to the genera Lactococcus which has several health benefits on infant's immune system [17]. *L. lactis* are mostly used as starter cultures in dairy industries due to its wide range

of applications [16] The variability in the strains used in dairy fermentation is very low. Majority of the bacteria strains used as starter culture in fermentation can be classified into small number of genetic lineages and are not the representatives of diversity found in L. lactis [8]. Therefore, finding of new strain of *L. lactis* is the necessity of time. *L. lactis* are also found on the surface of plants. But these plant derived strains of *L. lactis* have few low capabilities of showing good antimicrobial responses against human pathogens [9]. Strains to *L. lactis* isolated from HC have wide range of applications and have good potential of producing bacteriocins which inhibits the growth of other pathogens [10]. In the current study, *L. lactis* was isolated from HC and was genotypically and phenotypically characterized.

MATERIAL AND METHODS

Collection of Colostrum Samples:

About 4 different HC samples were aseptically collected from healthy lactating mothers who voluntarily offered to become a part of our study. Ethical clearance certificate from Institutional Ethics Committee was sorted before starting the study. The samples were collected aseptically in sterile tubes in presence of clinical experts. First few drops of colostrum was discarded to avoid contamination from skin flora and the mid-flow was carefully collected in vials. Samples were immediately transferred to laboratory for their proceedings.

Isolation of LAB:

HC samples were serially diluted upto 10^{-6} in 1% w/v sterile peptone water immediately after sample collection. The last three dilutions (10^{-4}, 10^{-5}& 10^{-6}) were used for inoculation. 0.1 mL of dilution was spreaded on the entire surface of MRS (deMan Rogosa Sharpe) [HiMedia] agar plates using sterile L-shaped spreader (Spread plate technique). The plates were incubated at 37°C for 24-48 h under anaerobic conditions using anaerobic gas jar.

Gram's Staining:

After incubation, the plates were observed for colony characteristics and the distinct colonies were further sub-cultured on MRS agar plates. The pure form of colonies was further used for Gram's staining. A drop of sterile water was placed on a clean glass slide and a pure colony was mixed gently to prepare a smear. The smear was heat fixed carefully at medium flame and further the standard procedure of Gram staining was followed. The slide was observed under phase contrast microscopy using oil emulsion. All the isolates which were observed Gram positive were further checked for their catalase activity.

Catalase Test:

After Gram staining, the isolates were checked for their catalase activity. A drop of 3% H_2O_2 was dropped on a clean glass slide and a pure colony was gently mixed on the surface of slide. The slide was observed for 30 seconds for formation of gas bubbles. All the isolates which did not showed catalase activity were further checked for their ability to ferment different sugars.

Sugar Fermentation Test:

The isolates which were found gram positive and catalase negative were further checked for their ability to ferment different sugars. 9 different sugars such as glucose, lactose, maltose, galactose, mannitol, sucrose, xylose, fructose and cellulose were prepared using standard protocols [HiMedia]. Durham's tube was added to each sugar for observing gas production. 0.1 ml of active cultures of LAB was inoculated in all different sugars and was incubated at 37°C for 24-48 h.

Primary Identification of Isolates:

On the basis of their biochemical characteristics, the isolates were identified using Bergey's Manual of Systematic Bacteriology (2nd Edition 3rd Volume).

Determination of Probiotic Activities:

To determine the probiotic activities, the isolates were tested for their growth at low PH, growth different bile salt concentrations, ability to resist against antibiotics and its antimicrobial activity.

Growth at low pH:

It is believed that the food eaten by us remains in stomach for about 4 h [15]. Therefore, all the isolates were inoculated in modified MRS broth prepared of different pH (2,3, 4, 5 and 6) and were incubated for 5 h. The isolates were then inoculated on MRS agar plates using spread plated method and the plates were incubated at 37°C in anaerobic gas jar. The isolates which gave good growth at low pH were further screened for checking their growth at different bile salt concentrations.

Growth at different bile concentrations:

According to the literature, the bile salt concentration in the intestine ranges humans ranges between 0.3 to 0.4% [15]. Therefore, all the isolates that showed positive growth at low pH were further inoculated in MRS broth prepared by adding different concentrations of bile salts (0.2, 0.3 and 0.4%). The tubes were allowed to incubate for 8 h after inoculation. The isolates were further inoculated on MRS agar plates and were incubated at 37°C in anaerobic gas jar.

Resistance to antibiotics:
The isolates which showed good growth at low pH as well as at different bile concentrations were checked for their resistance against different common antibiotics. Kirby Bauyer method (Disc diffusion method) was used for the study. The isolates were inoculated on Muller Hilton [HiMedia] agar plates using spread plate technique and the disc of antibiotics with different concentrations were placed on the surface of agar and were gently pressed. The plates were incubated in upright position at 37°C for 24-48 h and were observed for zone of inhibition.

Antimicrobial activity against pathogens:
The isolates which showed resistance against antibiotics were further tested for their activity against common human pathogens. Agar well diffusion method was used for this parameter. Plate was inoculated with overnight grown active cultures of pathogenic bacteria using spread plate technique. A sterile core borer was used to prepare wells on plate. The active cultures of isolates were poured in the wells and the plates were allowed to incubate in upright conditions at 37°C. The plates were observed for zone of inhibition.

Isolation of Bacterial DNA:
Out of all the isolates, *L. lactis* was found to have best probiotic potentials. Therefore, further molecular studies were performed for it. DNA was extracted using overnight grown liquid culture of *L. lactis* in MRS broth. 1 ml of pure liquid culture was centrifuged at 10000 rpm for 3 minutes. The supernatant was discarded and the pellet was used for DNA extraction. EXpure Microbial DNA isolation kit [Bogar Bio Bees Stores Pvt. Ltd.] was used. Standard protocols as per kit were followed for DNA isolation.

PCR amplification of 16S rDNA:
For amplification of 16S rDNA gene, the forward primer used was 27F with 20 number of base pairs 5' AGAGTTTGATCTGGCTCAG 3' and the reverse primer used was 1492R with 20 number of base 5' TACGGTACCTTGTTACGACTT 3'. PCR reaction mixture was prepared using 2X Taq DNA polymerase, 0.4mM dNTPs, 3.2mM MgCl2 and 0.02% bromophenol blue.5 µl of isolated DNA was added to 20 µl of PCR reaction solution. The amplification of reaction was carried out using thermal cycler (Bio-Rad).

Purification of PCR Products:
By using Montage PCR Clean up kit (Millipore), the unincorporated PCR primers and dNTPs were removed carefully. The protocol steps mentioned in the manual of kit was strictly followed. The purified PCR products were sequenced using primers. Sequencing was performed using ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing of Purified PCR Product:
Using 16s rRNA universal primers, Single-pass sequencing was performed on each template. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The sample was resuspended in sterile distilled water and was subjected to electrophoresis in an ABI 3730x1 sequencer (Applied Biosystems).

Phylogenic analysis:
The 16s rRNA sequence obtained was blast using NCBI Blast similarity search tool. The phylogenetic analysis of the query sequence with the other closely related sequence of blast was performed following the multiple sequence alignment. Multiple alignment of sequence was performed using a program called MUSCLE 3.7 [11]. The curing of result sequences was done through a program called Gblocks 0.91b. The main function of this Gblocks is to eliminate the poorly aligned positions and removes alignment noise [12,19]. At last, for analysing the phylogeny PhyML 3.0 aLRT was used and HKY85 was used as substitution model. PhyML was found to be as accurate as other programs. Tree Dyn was used for preparing phylogenic tree [13].

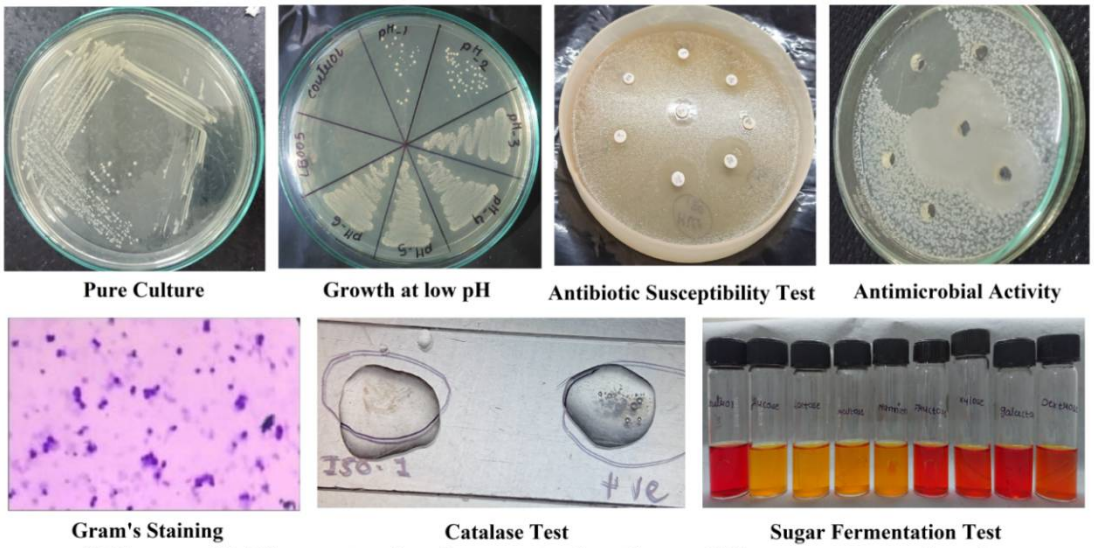
RESULTS

4 different HC samples were processed in the current study for isolating LAB. On inoculation of these samples on MRS agar plates, 7 isolates of LAB were isolated. On sub-culturing of these distinct colonies, 7 different LAB species were confirmed on the basis of their biochemical characterization [Bergey's Manual of Systematic Bacteriology (2nd edition 3rd volume)] . These species of LAB were further tested for their various probiotic potentials as mentioned above. Out of which, *Lactococcus lactis* was found to be very promising in showing best results in all the circumstances. The strain of *L. lactis* was able to survive at low pH as well as it was able to tolerant bile salts. The strain was also resistant to many of the antibiotics such as penicillin, ampicillin, etc. The strain of *L. lactis* also showed best antimicrobial activity and pathogen microorganisms [Figure 1]. Therefore, *L.lactis* was further selected for molecular characterization. The DNA of L. lactis was extracted and was multiplied using PCR technique. The PCR products were than purified and were processed for 16s rRNA sequencing as mentioned above. The

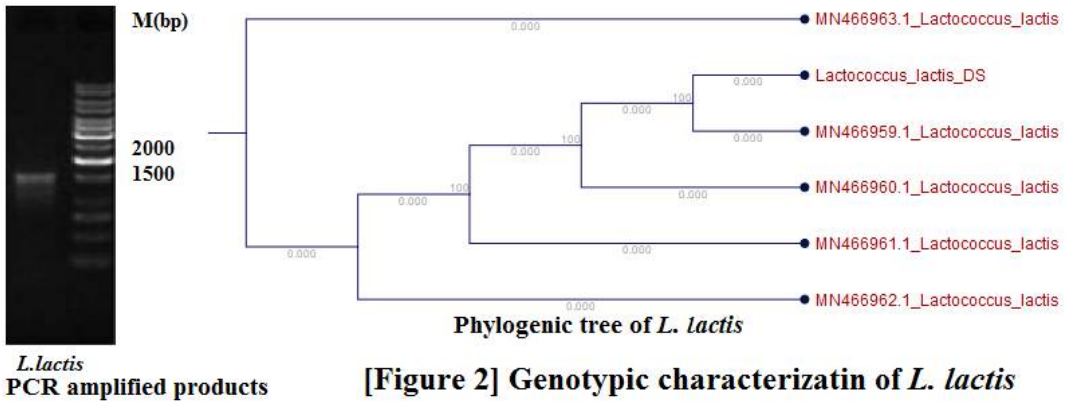
sequence of *L. lactis* was further analysed for its phylogeny [Figure 2]. According to the results of sequencing and phylogenic studies, *L. lactis* showed 100 % similarity with several other strains of *L. lactis.*

Table 1: Phenotypic characteristics and probiotic properties of *L. lactis.*

Phenotypic Characteristics	
Shape	Circular
Elevation	Convex
Margin	Entire
Texture	Shiny
Color	Light grey
Opacity	Opaque
Size	Moderate
Biochemical Test	
Gram's Staining	Positive
Cell Morphology	Cocci
Cell Size	1.2 μm
Catalase test	Negative
Oxidase test	Negative
Sugar Fermentation test	**Acid & Gas production**
Glucose	+ +
Maltose	+ +
Gluconate	- -
Mannitol	+ -
Glycogen	- -
Fructose	+ +
Sucrose	+ -
Xylose	+ +
Starch	- -
Lactose	+ -
Sorbitol	- -
Galactose	+ +
Esculin	- -
Cellobiose	+ -
Probiotic Estimation	
Growth at low pH	
Growth at pH 6	+
Growth at pH 5	+
Growth at pH 4	+
Growth at pH 3	+
Growth at pH 2	+
Bile Salt Tolerance	
0.2% w/v	+
0.3% w/v	+
0.4% w/v	+
0.5% w/v	+
Antibiotic Susceptibility Test (mcg/disc)	**Zone of inhibition in (mm) / R | I | S**
Trimethoprime (TR-30)	14 mm / I
Gentamycin (HLG-20)	27 mm / S
Amoxicillin (AMX-10)	08 mm / R
Penicillin G (P-1)	02 mm / R
Ceftazidime (CAZ-30)	09 mm / R
Ciprofloxacin (CIP-5)	08 mm / R
Streptomycin (HLS-300)	25 mm / S
Erythromycin (E-5)	03 mm / R
Anti-microbial activity	**Zone of inhibition in (mm)**
Staphylococcus aureus ATCC25923,	24 mm
Pseudomonas aeruginosa ATCC27853	09 mm
Salmonella typhi MTCC733	16 mm
Escheria coli ATCC25922	22 mm
Klebsella pneumonia MTCC3384	08 mm
Proteus vulgaris ATCC33420	11 mm



[Figure 1] Phenotypic characterization of *Lactococcus lactis*.



[Figure 2] Genotypic characterizatin of *L. lactis*

Sequence of *L. lactis*:

GGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATACCGCATAACA
ACTTTAAACATAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGT
TGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGA
CACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCG
CGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAGCTCAT
CAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCG
TTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCA
TTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCG
TAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAAGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGATGTAGGGAGCTATAAGTTCT
CTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACT
CGTGCTATTCCTAGAGATAGGAAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTA
ACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCATTCTCAGT
TCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTGCCTAACCGC

DISCUSSION

Seven different species of lactic acid bacteria were studied pheno-typically which were isolated from the colostrums four different lactating mothers. Only one strain of *L. lactis* was studied geno-typically. This strain was found to be with high probiotic potentials. Rodriguez and his team isolated about169 strains of lactic acid bacteria from the feces sample of infant [1]. Out of which 52 isolates were able to show antimicrobial response against *E. coli* and *Listeria innocua.* In 2012, Savitha and Malini reported the isolation of about 135 strains from sausages, paneer and cheese which belonged to the genera Bifidobacterium, Lactobacillus, Pediococcus and Lactococcus. The highest antimicrobial activity of *L. lactis* against S. aureus was reported [14]. In our study, *L. lactis* was able to show antimicrobial response against 6 different strains of pathogens. Large number of studies in the past have isolated lactic acid baceria from cow's, goat, buffalo, sheep and camel milk, but very less studies on isolation of lactic acid bacteria from human colostrum has been reported till date.

CONCLUSION

HC samples were found to be a rich source of probiotic LAB isolation. *L. lactis* was found to be the best among other isolated species of LAB. According to the present study, *L. lactis* is found to be the best strain with high probiotic potentials. This strain might have several health benefits and can be commercialized to cure the deficiency of probiotics in humans. For this, further In vivo studies are still required to be carried out to clearly justify the health benefits of *L. lactis.*

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