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RESEARCH ARTICLE

Characterization and identification of some probiotic properties of lactic acid bacteria isolated from curdled cow and goat milk

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Abstract: Since ancient times, curd is the most common fermented milk product consumed in India. Curd comprises brilliant gut healers that improves immune system. Raw milk of cow and goat is rich in various nutrients, minerals and vitamins. Additionally, it also contains mixture of probiotic lactic acid bacteria (LAB). The widespread use of synthetic antimicrobial agents, such as antibiotics, antifungals, and antivirals, can lead to the development of resistance in infectious microbes. This phenomenon is commonly referred to as antimicrobial resistance (AMR). For this reason, identification of natural antimicrobials like LAB appears to be essential for prevention and treatment. The purpose of this article is to isolate LAB from curd made from cow and goat milk and identify eminent probiotic LAB isolates which acquire safety aspects. The raw milk samples of cow and goat were allowed to curdle naturally and used for isolation of LAB. The gram positive and catalase negative LAB were isolated from curd samples. A total of 22 isolates were presumed as LAB form the curdled milk samples collected aseptically. Primarily these isolates were screened for cultural, microscopic and biochemical characteristics. Selected LAB strains were exposed to in vitro gastrointestinal conditions like low pH, bile salt, fluctuating NaCl and phenol conditions. Also, these isolated strains were evaluated for survival in stimulated gastric juice and stimulated intestinal juice. Further, safety property of these isolates was checked by their hemolytic, deoxyribonuclease (DNase) activity and susceptibility to antibiotics. On the basis of the evaluated results, LAB strains showing notable probiotic properties were further subjected to molecular identification. In conclusion *Lactiplantibacillus plajomi, Enterococcus italicus* and *Lactobacillus pentosus* are identified as LAB possessing impressing probiotic properties.

Keywords: LAB, probiotic, isolation, stimulated gastrointestinal conditions, safety property, molecular identification.

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Introduction

In India Cow and goat milk is consumed since olden times due to its quality of having rich nutrients, it is also considered to be a healing agent and is used widely for medicinal benefits. Cow and goat's milk almost rank similar in mineral content but both have different aids, combination of raw cow and goat milk may fulfill all the downsides lacking (Zhang, Lai et al. 2022) and (Mittu and Girdhar 2015). Lactic acid bacteria (LAB) are noble source of Probiotics i.e., live microorganism which on consumption in adequate quantity can confer apparent health benefits. They are well known for their medicinal properties. LAB was found significantly higher in raw milk as compared to pasteurized milk and higher macronutrients could be considered as one of the factors for the survival of LAB. LAB dominant in small and large intestine can inhibit the growth of pathogenic microbes by producing organic acids, bacteriocins and hydrogen peroxides. LAB stabilizes intestinal microflora after a long use of antibiotics LAB improves absorption of nutrients in gastrointestinal tract and decreases lactose intolerance. LAB in milk use enzymes to produce ATP from lactose, meanwhile by-product obtained during ATP production is Lactic acid that curdles milk and forms curd (Liptáková, Matejčeková et al. 2017) (König, Fröhlich et al. 2017) and (Zhang, Lai et al. 2022).

LABs are gram-positive bacteria (cocci, rods or coco bacilli) which does not form spores, non- pathogenic and typically are non-respiring but aerotolerant, fastidious, acid tolerant and catalase negative. LAB ferment carbohydrates and metabolically yields lactic acid as the key product during fermentation and other organic acids by products (Ibrahim, Naufalin et al. 2021).

Widespread use of synthetic antimicrobial agents causes resistance of infectious microbes to these compounds. For this

reason, identification of natural antimicrobials like LAB appears to be essential for prevention and treatment. New methods have been developed for preventing various multidrug This article documents the isolation of probiotic Lactic acid bacteria from cow and goat milk and its physiological, molecular identification and characterization.

Materials and methods

Collection and enrichment of samples

Fresh goat and cow milk samples were collected from different local milk farms of Surat district (latitude 21.170240 and longitude 72.831062), in sterilized autoclavable plastic bottles.

Within 3 hours of collection Milk samples were brought to the laboratory and stored at -2 to -4°C before the experiments. Goat milk was mixed with the cow milk at a ratio of 100:0 (T1); 50:50 (T2) and 0:100 (T3) and heated up to 40-45°C for 10 minutes to promote growth of bacteria as reported by (Temerbayeva et al. 2018)

All above test samples were incubated at 37°C overnight for curdling of milk by Lactic acid bacteria present naturally in raw milk samples. No starter culture is added, Prior to isolation of Lactic acid bacteria all samples were subjected to Enrichment step. Where, 0.1ml of homogenized sample was suspended to sterile de Mann Rogosa and Shapre broth (MRS) media (Hi-Media, India) and incubated at 37° C for 48 hours.

Isolation of LAB

The enriched MRS broth with samples were further streaked on sterile MRS medium and incubated in microaerophilic conditions at 37° C for 24 hours. The colonies with calcified zone were selected and further sub cultured onto MRS agar plates. Selected LAB isolates were maintained at -20°C in glycerol stock for further analysis.

Physiological and Biochemical identification of LAB

The isolates were subjected to preliminary screening to confirm whether the isolated colonies belong to the group of lactic acid bacteria. Lactic acid bacteria are known to give creamy white distinct round colonies on MRS media. According to (Sadia et al. 2021 and Somashekaraiah et al. 2019) LAB are gram positive, non-motile, non-sporulating, catalase negative isolates that were further subjected to biochemical analysis; Such as Simmon citrate, Indole, Methyl red, Voges Proskauer, Oxidase test, Urea hydrolysis and Carbohydrate fermentation test as recommended in Bergey's Manual of Determinative Bacteriology.

Evaluation of Probiotic properties

Tolerance to Low pH

The tolerance of isolated LAB strain to low pH is very important criteria for screening of probiotic organism. Staying time of food in stomach is approximately 3 h at 3 pH. 24 h old culture of selected LAB was inoculated in Phosphate Buffer Saline (PBS) of pH 2, 3 and 4 respectively using 0.1N Hydrochloric acid.

0.1 ml of suspension from respective PBS inoculated was transferred to sterile MRS broth after the time interval of 0, 1, 2 and 3 h of inoculation. These MRS broths were incubated at 37° C for 24 hours. Growth of LAB was monitored at OD_{620} as suggested by (Shaikh et al. 2013 and Estifanos et al. 2014).

Tolerance to Bile salt

The mean concentration of intestine is supposed to be 0.3% and staying time of food is suggested to be 4h. Fresh cultures of selected LAB strain were inoculated in PBS containing 0.2%, 0.3%, 0.4% Bile salts. Inoculation of 0.1 ml from this inoculated PBS was carried out after interval of 0, 1, 2, 3 and 4 h into sterile MRS broth. Further inoculated broth tubes were incubated at 37° C for 24 hours. Growth was observed by measuring absorbance at OD_{620} (Shaikh et al. 2013; Estifanos 2014).

Tolerance to NaCl

The isolates were incubated overnight in MRS broth for 24 h and harvested by centrifugation (7000 rpm,4°C,10 min). Sterile MRS broth of different NaCl concentration range (0-6%) were inoculated with 0.1 ml of selected LAB strains and incubated at 37° C for 24 hours. After incubation growth was monitored at OD_{620}. (Shaikh et al. 2013; Prabhurajeshwar et al. 2019)

Tolerance to Phenol

Determination of ability of LAB to resist phenol was carried out by inoculation of 0.1 ml of overnight grown LAB isolates in MRS broth supplemented with 0.4% and 0.6% v/v phenol (Shaikh et al. 2013; Somashekaraiah et al. 2019). Growth was observed by measuring absorbance at OD_{620} after incubation at 37° C for 24 hours.

Response to Stimulated Gastric Juice (SGJ)

Tolerance of LAB strain to stimulated gastric conditions was estimated by using the stimulated gastric solution that consists of Pepsin 3g/L at pH 2.5 using 0.1 N HCL (Ortakci et al. 2012) in sterile saline water (0.85%NaCl). Sterile SGJ was inoculated with overnight grown LAB isolates suspensions adjusted to 0.5 McFarland Standard and incubated at 37° C for 3h. MRS broth were inoculated with 0.1ml suspension from inoculated SGJ at 0 and 3h in an orbital shaker at 200 rpm to stimulate Peristalsis and incubated at 37° C for 24 hours. After incubation growth was monitored at OD_{620}. (Somashekaraiah et al. 2019 and Prabhurajeshwar et al. 2019).

Response to Stimulated Intestinal Juice (SIJ)

SIJ was prepared by mixing pancreatin 1g/L and Bile salt 0.03g/L at pH 8 using 0.1 N NaOH (Musikasang et al. 2009; Asan et al. 2018) in sterile saline water (0.85%NaCl). A pancreatin solution was inoculated with overnight grown LAB isolates suspensions adjusted to 0.5 McFarland Standard and incubated at 37° C for 3h. After 0 and 3 h 0.1 ml SIJ solution maintained at 200 rpm on shaker was inoculated in MRS broth. Further incubation and growth were monitored same as SGJ (Somashekaraiah et al. 2019).

Evaluation of safe probiotic strains

Haemolytic Activity

For assessment of Haemolytic activity of LAB isolates 5% (w/v) Sheep blood agar plate (Somashekaraiah et al. 2019) was streaked with overnight grown cultures of selected LAB and incubated at 37°C for 2- 3 days. After incubation plates were then evaluated for the haemolytic reaction i.e. á haemolysis- green zone or â haemolysis- clear zone and ã haemolysis no clear zone around the colonies on blood agar plates. (Yadav et al. 2016)

DNase Activity

To check the production of DNase enzyme LAB isolates were streaked on DNase agar medium (HiMedia, India). Plates were then incubated at 37° C for 24-48 h. After incubation plates were observed for DNase activity. (Monique et al. 2020) reported that colonies surrounding with clear or a pinkish zone was considered as positive.

Antibiotic Susceptibility

LAB isolates were assessed for their resistance to antibiotics by Disc Diffusion method by using antibiotic discs of Streptomycin, Tetracycline, Erythromycin, Penicillin, Kanamycin, Chloramphenicol, Amikacin, Ampicillin and Clindamycin. This method was originally standardized as per ISO 10932/IDF 233 standards (Erginkaya et al. 2018). Actively grown culture was swabbed on the MRS agar plates. Antibiotic discs (HiMedia, India) were placed on the inoculated agar surface with three replicates and then incubated at 37° C for 24-48 h. Diameter of zone of inhibition around colonies were measured using Vernier caliper (Luana et al. 2014).

Molecular Identification of LAB

Identification of LAB isolates were carried out by 16s RNA gene sequencing using the primers 27F:52 - AGAGTTTGATYMTGGCTCAG and 1492R:52 - TACCTTGTTAYGACTT. This technique is fast and valid for molecular identification. The isolation of genomic DNA of LAB was done by using genomic purification kit (HiMedia, India). The quality of isolated DNA from overnight grown culture was evaluated on 1% Agarose gel, Gene fragments were amplified by PCR (polymerase chain reaction) and PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was performed with 16s BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. The resultant sequences were examined for similarity in the database of NCBI GeneBank using nBLAST (www.ncbi.nlm.nih.gov/blast). The obtained sequences were submitted to the GeneBank for accession numbers. The Evolutionary analyses were conducted in Mega6. (Rine et al. 2019).

Results and Discussion

Isolation and Identification

A total of 71 bacterial cultures were initially isolated from naturally curdled raw milk samples collected. These isolates were further proceeded to physiological and biochemical tests. Out of total isolates 22 of them were presumed as Latic acid bacteria on the basis of their morphological and colony characteristics. Only

Table 1: Morphological characteristics of LAB

Isolates	Gram character	Motility	Endospore staining	Capsule staining	Colony characteristics		
					Size	Shape	Color
M5	+, rods	-	-	-	Small	Round	Cream
M8	+, rods	-	-	-	Small	Round	White
M9	+, rods	-	-	-	Pin point	Round	Cream
M13	+, rods	-	-	-	Medium	Round	White
M16	+,cocco-bacilli	-	-	-	Large	Irregular	Dew drop
M17	+, rods	-	-	-	Small	Round	Cream
M20	-, rods	-	-	-	Large	Round	White
M27	+, rods	-	-	-	Small	Round	Cream

-Positive and +Negative

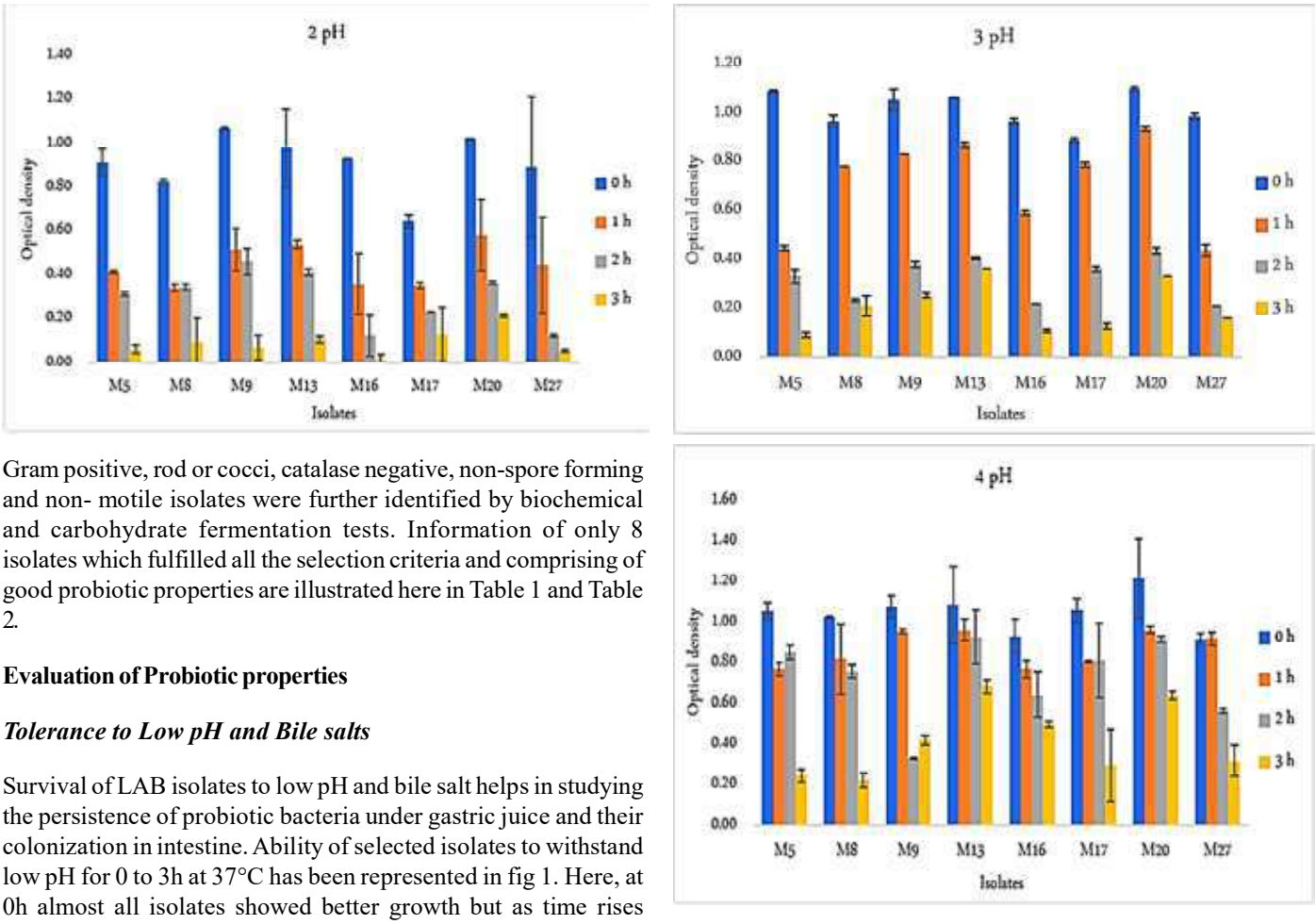


Fig 1. Growth of Isolates M5, M8, M9, M13, M16, M17, M20 and M27 at 2 pH, 3 pH and 4 pH at 0h, 1h, 2h and 3h were observed by measuring absorbance at OD_{620}. Values are given as mean = SD(n=3).

Gram positive, rod or cocci, catalase negative, non-spore forming and non- motile isolates were further identified by biochemical and carbohydrate fermentation tests. Information of only 8 isolates which fulfilled all the selection criteria and comprising of good probiotic properties are illustrated here in Table 1 and Table 2.

Evaluation of Probiotic properties

Tolerance to Low pH and Bile salts

Survival of LAB isolates to low pH and bile salt helps in studying the persistence of probiotic bacteria under gastric juice and their colonization in intestine. Ability of selected isolates to withstand low pH for 0 to 3h at 37°C has been represented in fig 1. Here, at 0h almost all isolates showed better growth but as time rises proliferation of M9, M13 and M20 showed improved viability. And survival of LAB cultures in bile salts for 4h at 37°C mentioned in fig 2. Overall, among all the isolates tested, M9, M13, and M20 exhibited higher survival rates compared to the others. (Parisa S., et al. 2014)

Tolerance to NaCl

Almost all isolates showed significant growth but notable sustainability was observed in M13 and M20 for 24h. Thus, it can be observed that all isolates displayed resistance at various NaCl concentrations. The results have been shown in fig 3.

Tolerance to Phenol

Isolates showing resistance to above gastric conditions were further subjected to phenol resistance at concentration 0.4% and 0.6%. Results are displayed in fig 4. Isolates M9, M13 and M20 showed good tolerance and less sensitive to phenol concentration 0.4% and 0.6%. Whereas, M5, M8, M16, M17, M20 and M27 were more sensitive.

Tolerance to Stimulated Gastric and Intestinal juices

Survival in stimulated gastrointestinal conditions is very important criteria for selection of potential probiotic isolates. Lactic acid bacteria showing highest resistance to above gastric conditions were further subjected to evaluation for in vitro resistance to simulated gastrointestinal environment. As per results presented in fig 5. at 0h growth of all isolates is observed but after 3h of incubation only M9, M13, M20 and M27 showed higher tolerance to stimulated gastric juice and intestinal juice compared to M5, M8, M16 and M17.

Evaluation of safe probiotic strains

Haemolytic Activity

For the selection of safe probiotic strain safety properties of selected isolates is a principal measure. It is obligatory to evaluate invitro analysis of haemolytic activity on blood agar plate. The results exhibited that all selected LAB strains were ã haemolytic. Strain M5, M8 and M17 showed non hemolysis i.e. no clear zone

around the colonies on blood agar plate.

DNase Activity

The DNase plates inoculated with LAB isolates showed no zone around the colonies which confirms and proves that none of the LAB strains has ability to produce DNase enzyme. Hence, they may be non-pathogenic probiotic isolates; however, for safe use in humans and animals, in vitro analysis should be conducted.

Antibiotic Susceptibility

Table 2: Biochemical and Carbohydrate fermentation test of LAB

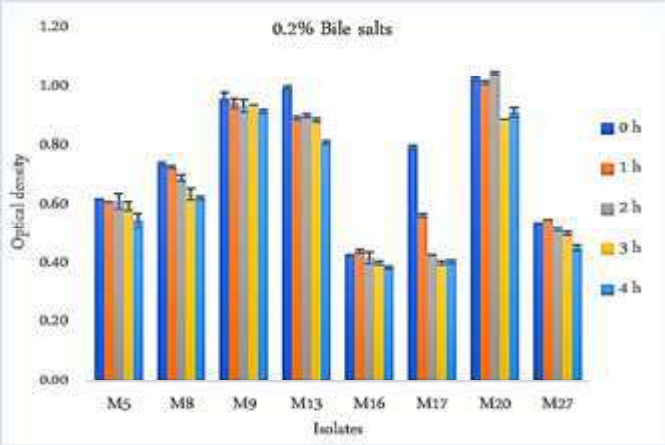
Parameters / Biochemical tests	Isolates							
Catalase test	M5	M8	M9	M13	M16	M17	M20	M27
Oxidase test	-	-	-	-	-	-	-	-
Citrate utilization Test	-	-	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-	-	-
Voges prausker Test	-	-	-	-	-	-	-	-
Urea hydrolysis Test	-	-	-	-	-	-	-	-
Peptone utilization test	-	-	-	-	-	-	-	-
Gelatin hydrolysis Test	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-
Carbohydrate fermentation test								
Glucose	-	+	+	±	±	-	-	-
Fructose	+	+	+	+	+	+	±	±
Sucrose	+	±	+	+	±	±	±	-
Maltose	-	+	+	+	+	+	+	+
Mannitol	+	+	-	+	+	+	-	+
Lactose	+	-	-	+	-	-	+	±
Xylose	+	+	-	+	+	+	±	±

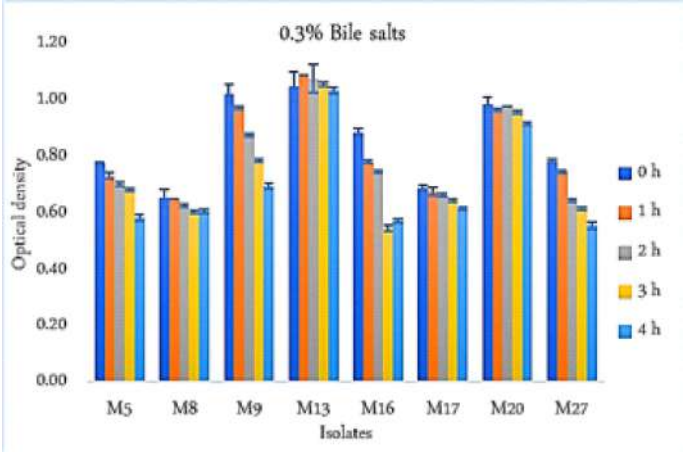
-Positive, +Negative and ± Variable

Table 3: Antibiotic Susceptibility test

Antibiotic	Concentration (µg/disc)	M5	M8	M9	M13	M16	M17	M20	M27
Chloramphenicol	30	S	S	S	S	S	MS	S	S
Amikacin	30	R	S	S	MS	R	S	MS	R
Amphicillin	10	S	S	S	R	S	S	S	MS
Erytromycin	15	R	S	MS	R	S	S	S	MS
Streptomycin	25	S	S	S	S	S	MS	MS	S
Kannamycin	30	R	R	R	R	R	R	R	R
Penicillin	10	S	S	S	S	S	S	S	S
Clindamycin	20	S	S	S	S	S	R	S	S
Co Trimoxazole	25	S	S	MS	R	MS	S	S	S
Tetracycline	30	MS	S	S	S	S	S	S	S

S- susceptible, R- resistant and MS- moderately susceptible





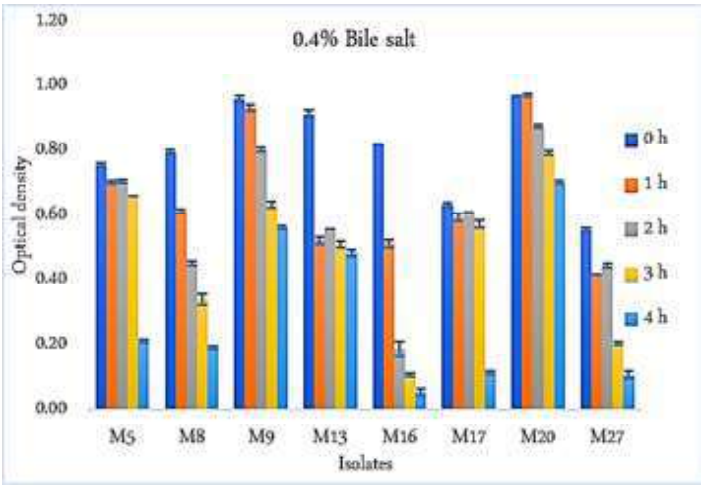


Fig 2. Growth of Isolates M5, M8, M9, M13, M16, M17, M20 and M27 at 0.2%, 0.3% and 0.4% bile salt concentration at 0h, 1h, 2h, 3h and 4h were observed by measuring absorbance at OD_{620}. Values are given as mean = SD(n=3).

One of the eminent probiotic property of a LAB strain is that it should be lacking antibiotic resistance. Any LAB must not carry any transportable antibiotic resistance gene that can be easily shifted to pathogenic organisms. Contrary to this, it is necessary for probiotic organisms to subsist in intestine during antibiotic treatments. Thus, inherent antibiotic resistant of LAB is considered natural and beneficial to host. As suggested by Morten et al. (2003) Two groups of antibiotics are recommended for selection of strain for instance antibiotics inhibiting cell wall synthesis (ampicillin and vancomycin) and antibiotics inhibiting protein synthesis (chloramphenicol, clindamycin, erythromycin, streptomycin, kanamycin and tetracycline). As per the results and diameter of zone measured were compared with the zone size interpretative chart (Hi-Media). Most of the isolates were sensitive to chloramphenicol, ampicillin, erythromycin, streptomycin, penicillin, clindamycin, co-trimoxazole and tetracycline and all isolates showed resistance to this Kanamycin as reported in (Table 3). The results attained are displayed in terms of susceptibility (S), moderate susceptibility (MS) and resistance (R). According to Elkins et al. (2004) most of the strains are resistant to Kanamycin and it has been reported earlier too, but it could be accreditable to the absence of cytochrome-mediated electro transport, which facilitates drug uptake. There is no trouble for low resistance towards kanamycin since the strains exhibited high susceptibility to clinically relevant antibiotics, so could be totally free of transferable antibiotic resistance gene.

Molecular Identification of LAB

Isolates showing renowned probiotic abilities were further identified by 16S r RNA gene sequencing and phylogenetic analysis as reported in (Table 4). On the basis of the results of Blast analysis it was found that 16S rRNA gene sequence of organism coded CG1 showed 98% similarity to *Lactiplantibacillus plajomi*, strain coded CG5 showed 100% similarity to *Enterococcus italicus*, strain coded CG13 showed 100% similarity to *Lactobacillus pentosus* sequence submitted to Gene Bank.

Table 4: Identification of the lactic acid bacterial isolates using 16S rRNA gene sequences

Isolates	Accession number	The nearest matched species from GenBank	Similarity %
CG9	OM 169332	*Lactiplantibacillus plajomi*	98
CG13	OM 670160	*Enterococuss italicus*	100
CG20	ON 495683	*Lactobacillus pentosus*	100

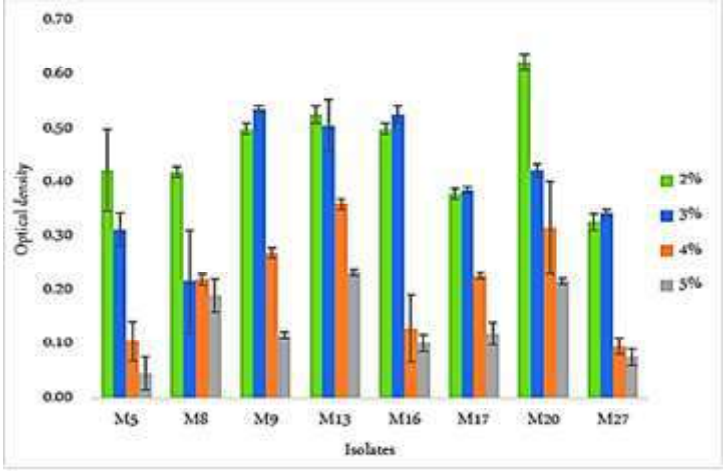


Fig 3. Growth of Isolates M5, M8, M9, M13, M16, M17, M20 and M27 at 2%, 3%, 4% and 5% salt concentration were observed by measuring absorbance at OD_{620}. Values are given as mean = SD(n=3).

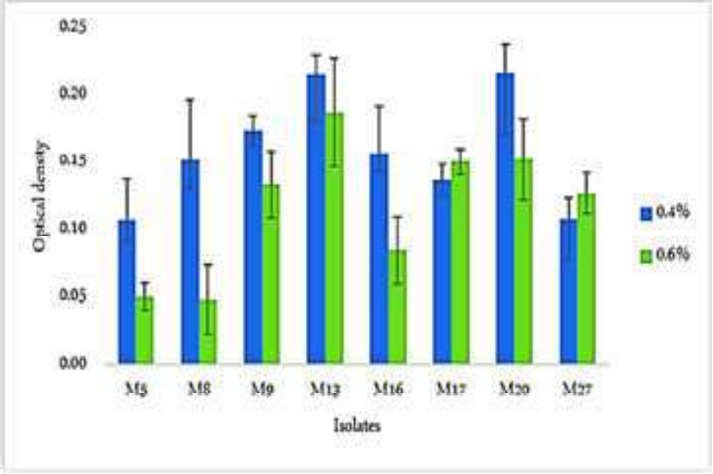


Fig 4. Growth of Isolates M5, M8, M9, M13, M16, M17, M20 and M27 at 0.4% and 0.6% phenol concentration were observed by measuring absorbance at OD_{620}. Values are given as mean = SD(n=3).

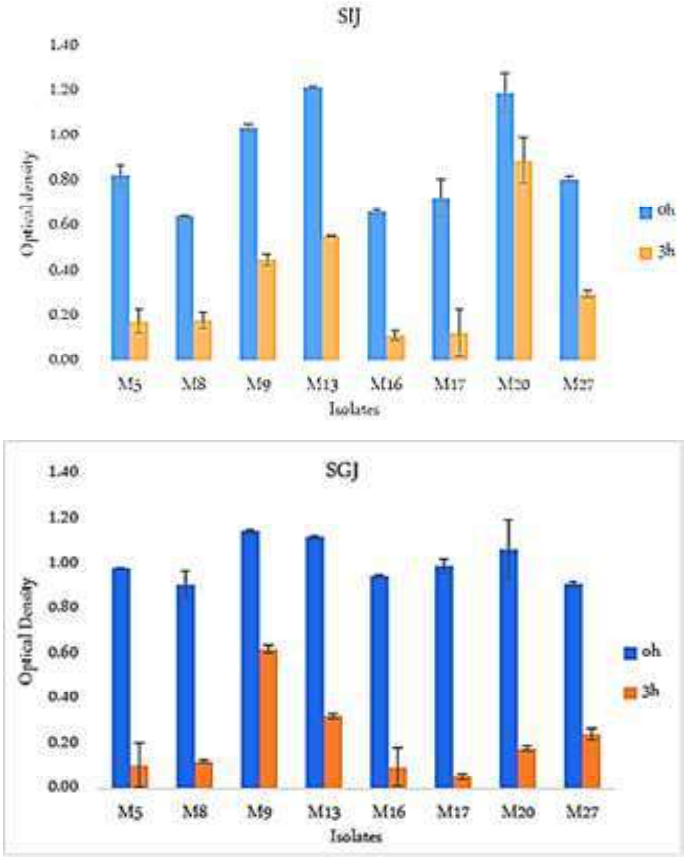


Fig 5. Growth of Isolates M5, M8, M9, M13, M16, M17, M20 and M27 in SGJ and SIJ at 0h and 3 h were observed by measuring absorbance at OD_{620}. Values are given as mean = SD(n=3).

Conclusion

This study focused on screening of probiotic lactic acid bacteria from naturally curdled milk of cow and goat. These probiotic strains were isolated and identified according to physiological and biochemical characteristics. All the LAB isolates could survive in GI tract but these three LAB isolated displayed striking tolerance to severe gastrointestinal conditions like resistance to low pH, bile salt, varying NaCl and phenol concentrations and survival in stimulated gastrointestinal conditions. Followed by susceptibility to several clinically effective antibiotics and evaluated as safe probiotic strains. Therefore, from these results we divulge that *in vitro* potential probiotic isolates *Lactiplantibacillus plajomi*, *Enterococcus italicus* and *Lactobacillus pentosus* are successfully isolated from naturally curdled cow and goat milk. However, further investigation can be performed to use these isolates reliably. Like many other safety parameters, *in vivo* assessments in animal models can be conducted. Further exploration of health benefits and potential applications can also be pursued.

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