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RESEARCH ARTICLE

Isolation and identification of potential probiotic lactic acid bacteria (*Lactobacillus casei* and *Bifidobacterium*) from raw and fermented camel milk during storage

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ABSTRACT

The aim of this study is to isolate and molecularly characterize LAB present in raw and fermented milk. The sample was collected in local areas in Jaipur Rajasthan. The identification of the selected LAB strains and their genetic relatedness was performed based on 16S rDNA gene sequence comparisons. They differed in their different probiotic characteristics such as tolerance to acidic pH, resistance to bile tolerance, and antibacterial activity. In conclusion, the isolates *Lactobacillus casei*, *Enterococcus durans*, *Lactobacillus Plantarum*, and *Bifidobacterium* were most probably high quality with probiotic potentials. We speculate studying the synergistic effects of bacterial combinations might result in a more effective probiotic potential, Lactobacilli species (42.90%). Enterococcus spp isolates represented 28.26%, The remaining isolates were *Lactobacillus casei* and *Bifidobacterium* that represented 4.87% and 2.42%. We suspect in raw and fermented camel milk was rich in LAB and has capable probiotic potential.

Keywords: Lactobacilli, LAB, Bile tolerance, 16S rDNA, *Bifidobacterium, probiotics*.

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INTRODUCTION

The potential Probiotic bacteria can confer human health benefits to the human gastrointestinal tract. Lactic acid bacteria (LAB) are candidate probiotic bacteria that are extensively distributed in nature and can be used in the food industry. Using LAB in food is one of the historically recognized food preserving techniques. LABs are extensively spread. It was observed in many food products such as dairy, meat, beverages, and vegetables, however, they are additionally current in the mouth, digestive system and vagina of mammals (Saeed and Ibrahim, 2013). Also, Lactic acid bacteria are an important part of the food industry, they are used for the health improvement, the manufacturing of macromolecules, metabolites, and enzymes.Lactic acid bacteria are a group of microaerophile or anaerobic gram-positive bacteria that are unable to form spores or produce catalase and are characterized through the absence of the cytochrome system and the capability to produce antimicrobials for preservation. Certain food, which includes dairy products, such as yoghurt, are good sources of probiotics. The majority of microbiota in raw and fermented milk products consists of the genera *Lactobacillus, Enterococcus, Lactococcus, Leuconostoc, Pediococcus, Oenococcus*, and *Streptococcus*. Bacteria in general, require appropriated

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biochemical and the environment to develop categorical normal metabolic activities. There are many elements that affecting the growth of LAB strains, consisting of the intrinsic food itself (nutrient content, water activity, pH value, antimicrobial and mechanical barriers to microbial invasion.) consisting of extrinsic associated to the environment (temperature of storage; the atmosphere surrounding the food). Also, the factors related to the microorganisms themselves and processing factors. (Hammam, 2019) and (Saeed and Ibrahim, 2013).

LAB is capable to produce bacteriocins and their consumption confers various health benefits, such as controlling intestinal infections, enhancing lactose utilization, decreasing blood ammonia levels, presenting efficient resistance against gastric acid and bile tolerance, influencing the immune system, and reducing serum cholesterol stages. Thus, the aim of this research is to evaluate the impact of whey camel's milk (that is antimicrobial proteins) on the reaction of lactic acid bacteria isolated from camel raw and fermented.

MATERIALS AND METHODS

Sample collection

Milk samples were collected from neighbourhood camels reproducing ranch in the State of Rajasthan will be used for isolating LAB probiotic bacteria. The sample will be permitted to ferment at room temperature for 1 week without any additives through the raw milk endogenous microorganisms.

Isolation and growth condition

The procedure of the collected sample was done as takes after a 10 mL volume of raw camel milk will be added to 80 mL MRS (deMan, Rogosa, and Sharpe) broth medium in 150 mL conical flasks. The enhanced sample was incubated at 30 °C for one week under static conditions. The high volume of the media gave appropriate conditions to the facultative anaerobic microorganisms and made it unnecessary to incubate the sample anaerobically. The enhancement procedure was conducted in triplicate and repeated every week for one month period. The isolation process was completed by the enriched sample on MRS agar media and the isolated bacteria were incubated at 37 °C anaerobic conditions for 24 hr. The isolated bacterial cultures were characterized and identified by using DNA sequencing methods. The identification method depends on 16S rRNA gene sequencing similarity. The raw sequencing information documents were aggregated into arrangements and sequence comparisons were obtained and analyzed using Applied Biosystems Microseq software or Genbank database libraries. The identification process was also confirmed by fatty acid methyl ester analysis. (Montgomery et al., 1999) and (Thomas et al., 1997).

Acidifying activity

Acidifying action of strains were estimated (Ki-Hal et al., 1999; Calleja et al., 2002). Acid production capacity was tested by inoculating 10 % skim milk with 24h old culture at 1% level and incubation at 30 °C. pH was determined during 24h of the incubation period.

Phenotypic characterization of active isolation

The isolation of lactic acid bacteria which delivered CFS to proteolytic catalysts (bacteriocins) was described by using the phenotypical and biochemical tests (Bradbury, 1997).

Lipolytic action

To decide the lipolytic activity, the strains were inoculated on an agar spot in Tween 80 (1, 3, 5%). Incubation was done at 25 °C for 72h. Strains with an obscure region because of the development of esters with calcium liberated unsaturated fats were viewed as positive. Lipolytic activity was resolved from the distance across the lytic zone (Briges, 1953).

Antibacterial effect

For the antibacterial movement test, the spot-on garden technique was used. 18h cultures were spotted on MRS agar plates and incubated for 24h at 37 °C under anaerobic conditions. Medium-term indicator strains (Listeria inocula, Micrococcus luteus, and Escherichia coli) were overlaid in delicate agar on MRS plates. Plates were incubated at 37oC for 18h at that point, restraint zone distances across were estimated. Ni sin (1mg/ml) will be used as the control.

Tolerance of Isolated LAB to Acidic pH

The tolerance of the isolated bacteria to acidic pH was executed as portrayed by (Gotcheva et al., 2002). Each bacterial isolate will develop in MRS (deMan Rogosa Sharpe) stock and incubate at 37 °C medium-term, at that point sub-cultured into crisp MRS broth and incubated for another 24 hours. The bacterial cultures were centrifuged at 5000 rpm for 10 min at 4 °C and the pellets were washed twice in sterile phosphate-buffered saline (PBS, 0.1 M phosphate support, 0.8% NaCl, pH 7.2) and re-suspended in PBS. Each strain was diluted 1/100 in PBS at pH 1.0, 2.0 and 3.0 and hatched for 1, 2 and 3 hours. Tallies of surviving bacterial colonies were resolved after plating the bacterial isolates on MRS agar with suitable pH and incubating them anaerobically at 37°C medium-term. Control tests without fermentation were additionally arranged and correspondingly dealt with Amplification and Sequencing of PCR bands

Agarose gel electrophoresis was carried out to visualize amplified DNA fragments and to excise corresponded bands with a sterile scalpel by using UV light after ethidium bromide staining. The amplicons of PCR were purified with Wizard PCR Preps DNA Purification and stored at -20 °C. Sequencing was completed by Eurofins Genomics enterprise. Sequence annotation and database searches for comparable sequences were carried out by the use of BLAST at the National Center for Biotechnology Information to decide the closest recognized relative species.

RESULTS AND DISCUSSION

The isolation of Lactic acid bacteria from natural sources has always been the most powerful means for obtaining beneficial and genetically stable strains for industry necessary products.

Morphological tests and biochemical reactions (Pre- identification)

In the study, 40 isolates that included 11 isolates were obtained from raw and fermented camel milk. Pre-identification of isolates of two different camel's milk samples are illustrated in (Table No. 1). Milk samples collected from a neighbourhood camels reproducing ranch in the State of Rajasthan will be used for isolating LAB probiotic bacteria.

Table 1: Identification Results Of Strains Isolated From Raw Camel Milk Samples

Strain no.	Pre Identification	Gram Stain	Oxidase	Catalase	Temp		Growth On 6.5% salt
					37°	42°	
1CM	Lactobacillus durans DCM 1008 16S	+	-	-			-
2CM	Lactobacillus casei sa strain BN11 16S	+	-	-			-
3CM	Lactobacillus plantarum DSM 9508 16S	+	-	-			-
4CM	Lactobacillus casei 16S isolate MG3	+	-	-			-
5CM	Lactobacillus futsaii Y8 16S	+	-	-			-
6CM	Lactobacillus sp. NLAE-zl-P160 16S	+	-	-			-
7CM	Lactobacillus strain E064 16S	+	-	-			-
8CM	Lactobacillus lactis S-P155 16S	+	-	-			-
9CM	Lactobacillus casei H248 16S	+	-	-			-
10CM	Lactobacillus acidophilus LBR1 16S	+	-	-			-
11CM	Lactobacillus acidophilus CPI 10426 16S	+	-	-			-

(CM =camel milk, Two Different Temperature)

All these strains were tested by Gram's staining, oxidase, and catalase tests. After laboratory screening, 40 strains were Gram positives, oxidase negative, and catalase-negative and non-spore-forming bacteria which were considered as lactic acid bacteria strains were isolated from 11 samples of camel's milk and 5 samples of raw camel milk. (Gomes et al.,2011). IThe isolates were differentiated according to their morphological and biological characteristics into five genera of LAB as follows: Enterococcus, Lactobacillus Plantarum, Lactobacillus case, Lactobacillus acidophilus, and Bifidobacterium. Isolated bacterial strains were classified into 3 groups by (Mahrous, 2013). group A, (obligate-homo- fermentative), B (facultative-hetero-fermentative), and C (obligate-hetero-fermentative).

The results were obtained from camel's milk that Lactobacilli species (42.90%). Enterococcus spp isolates represented 28.26%, The remaining isolates were Lactobacillus casei and Bifidobacteria that represented 4.87% and 2.42%. According to results, Enterococcus and Lactococcus genus seem to be dominating in camel's milk.

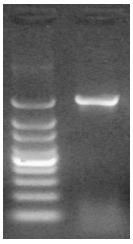

Figure 1. 1.2% Agarose gel showing single 1500 bp of 16S rDNA amplicon. Lane 1: 100bp DNA ladder; Lane 2: 16S rDNA amplicon.

Identification by 16S rDNA

Isolated DNA was amplified with 16S rRNA Specific Primer (8Fand 1492R) using Veriti® 99 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band of 1500 bp was observed in figure 1.

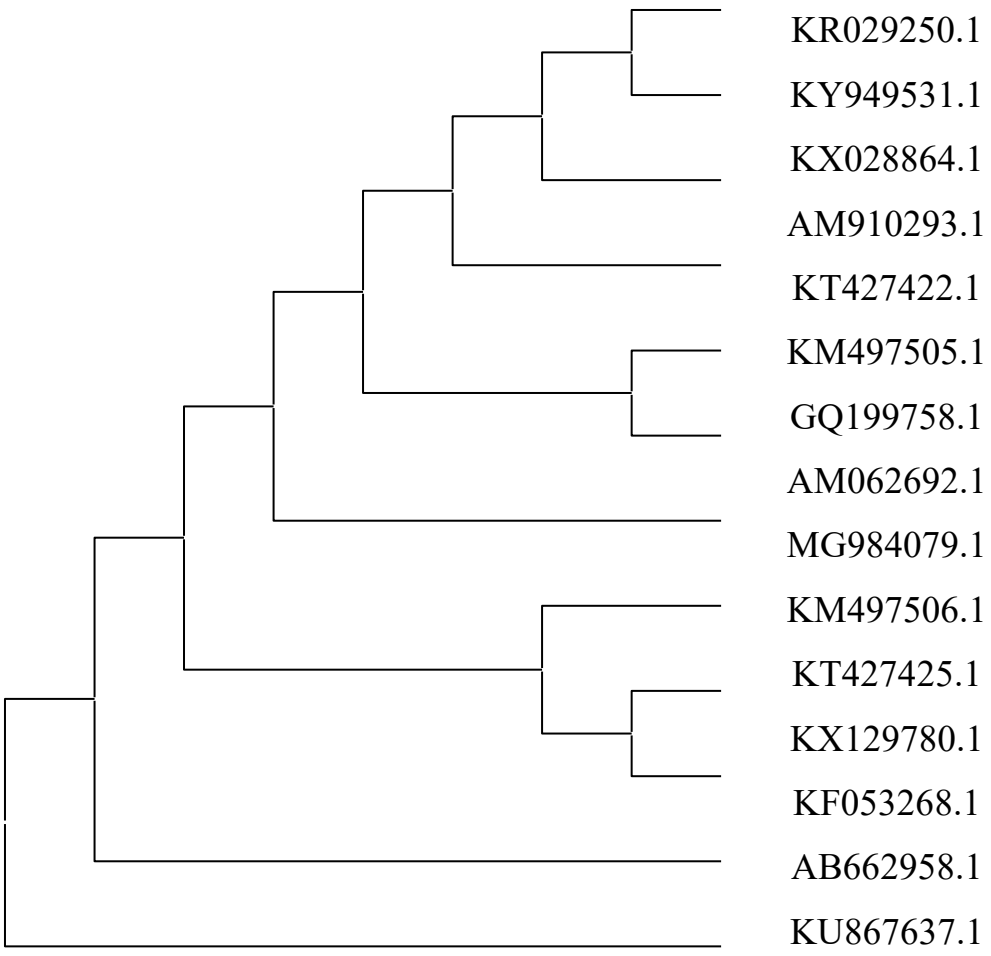

Figure 2: Phylogenetic analysis

The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. The 16S rDNA sequence was used to carry out the BLAST alignment search tool of the NCBI Genbank database. Based on the maximum identity score first 11 sequences were selected and aligned using the multiple alignment software program ClustalW. The distance matrix was generated using the RDP database and the Phylogenetic tree was constructed using MEGA5. The culture, which was labelled as M2 showed similarity with Lactobacillus casei 16S isolate MG3 based on nucleotide homology and Phylogenetic analysis shows that 11 isolates obtained from camel milk and one isolate obtained from camel milk were identified as Lactobacillus casei similarity level 96%, On the other hand, two isolates obtained from raw camel milk that were pre-identified as Lactobacillus acidophilus CPI 10426 16Sby PCR amplicon at 89% and 88% similarity level. Among Enterococcus 11 isolates obtained from camel milk and four isolates obtained from cow milk were identified as Enterococcus faecium (En. Faecium) using Rep-PCR amplification with a similarity level of 99% compared with CNRZ 131 as a reference strain. On the other hand, one isolate was identified as Bifidobacterium and represented as a minor species of LAB in camel milk This result was in agreement with (Savadogo *et al*., 2004). found detected Bifidobacterium species in traditional fermented camel milk and it represented 10% of LAB isolated from samples studied in (Table No 2).

Table 2: Identification Results Of Strains Isolated From fermented Camel Milk Samples

Accession	Description	Max Score	Total score	Query Coverage	E value	Max ident
NR_117155.1	Lactobacillus durans DCM 1008 16S	1055	1066	98%	0.0	88%
NR_104805.1	**Lactobacillus casei sa strain BN1116S**	1055	1066	98%	0.0	94%
NR_117378.1	Lactobacillus plantarum DSM 9508116S	1077	1044	98%	0.0	87%
LN881578.1	Lactobacillus casei 16S isolate MG3	1056	1035	91%	0.0	96%
KP119717.1	Lactobacillus futsaii Y8 16S	1055	1011	97%	0.0	87%
JQ607016.1	Lactobacillus sp. NLAE-zl-P160 16S	1011	1011	96%	0.0	87%
JX267093.1	Lactobacillus strain E064 16S	1011	1002	98%	0.0	91%
JQ607029.1	Lactobacillus lactis S-P155 16S	1000	1000	96%	0.0	87%
JX006468.1	Lactobacillus casei H248 16S	996	989	96%	0.0	94%
GQ461808.1	Lactobacillus acidophilus LBR1 16S	996	989	95%	0.0	87%
GQ461813.1	Lactobacillus acidophilus CPI 10426 116S	996	989	97%	0.0	89%

CONCLUSION

This study confirmed the behaviour of LAB isolated from camel milk that can grow in the complicated ecosystem like camel milk; it resists the camel milk whey that is highly present in antibacterial proteins. It is recommended that these species should be further studied according to selection criteria for dairy industries such as (EPS production, stimulation of immunological system acidifying activities, and the benefit from their unique characteristics to produce functional dairy products.

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