Research Article

Isolation of *Lactobacilli* from Freshly Drawn Raw Milk of Desi and Crossbred Cows of Gurugram Region, Haryana

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ABSTRACT

Background: Cow milk is of great concern these days because of its high nutritional value as well as the probiotic flora present in it, which confer several benefits to the human body. *Lactobacillus* is one such beneficial bacterium present in milk that helps the human body against allergic reactions, gastrointestinal diseases, and irritable bowel. **Aim:** The current study aims to determine the prevalence and isolation of *Lactobacillus* present in milk. The raw cow milk samples were collected from two categories of cows: desi cows and cross-breed cows. Raw cow milk is said to be harmful for human consumption due to the presence of pathogenic microflora. **Materials and Methods:** The raw cow milk samples were cultured on MRS agar (selective media) to isolate the diversity of Lactic acid bacteria (LAB) present in cow milk as probiotic flora. A total of twelve milk samples were taken; 5 from the cross-breed cows and 7 from the desi cows. Samples were cultured on MRS agar anaerobically for 24-48 hours. The bacterial isolates were tested for morphological characters, and biochemical tests including catalase, oxidase, citrate, sulfide, indole motility, and tolerance at physiological conditions like growth at different temperatures (ranging from 25- 450C), growth in different saline concentrations (2%, 4%, 6%, 8%, and 10%), and growth in different pH (4, 5, 6, 7, 8). **Result:** At the end of the study, three isolates were found gram-positive rods, out of which, 2 were catalase-negative and one was catalase-positive. All three strains were able to tolerate some physiological conditions. **Conclusion:** These can be further used as preservatives in some food items to increase their nutritional value as well as for further molecular studies for the identification of particular *Lactobacillus* species. Some new strategies should be implemented to make more efficient identification of LAB strains.

Keywords: Raw Milk, *Lactobacillus*, Probiotics, Natural Source, Biochemical Tests, Physiological Tolerance.

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INTRODUCTION

Milk and milk products provide a wealth of nutrition benefits. Food-borne pathogens are more prevalently found in dairy products like milk.[1] Raw milk or unpasteurized milk is more prone to be contaminated by bacterial pathogens.[2,3] Regulatory organizations like; Food and Drug Administration[4] and the Centre for Disease Control and Prevention (CDC) have raised important concerns on the risk of illnesses if the raw milk is consumed.[5] In dairy products, cow milk is considered a primary source of food for Lactic Acid Bacteria (LAB) out of which most of them act as Probiotics.[6] Probiotics are a group of living bacteria that possesses some health benefits to living organisms and these are safe to administer along with diet.[7] The presence of high counts of LAB in cow milk as salutary microbiota indicates a source for studies for its use in producing probiotic products at a large scale and the factors which affect the prevalence of LAB.[4] Also, if the microbial diversity of LAB in unpasteurized and pasteurized cow milk is compared then the results show more diversity

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in the pasteurized cow milk rather than in unpasteurized cow milk.[8,9] There have been many reports from North India where microbial contamination has been reported in the food chain including water, salads and meat samples.[10-14]

Typically, raw cow milk has the potential of containing diverse LAB species including *Lactococcus, Bifidobacterium, Pediococcus, Streptococcus, Lactobacillus, Leuconostoc, and Enterococcus* species.[15] Other than these, milk contains many pathogenic bacteria such as *Listeria, Salmonella, Escherichia coli, Campylobacter, Clostridium, and Pseudomonas* which lead to spoilage of milk and are responsible for pathogenesis in the human gut.[16] In recent years, the importance of administering probiotics as a dietary supplement is drawing attention to the scientific evidence that it benefits and maintains a healthy human gut flora.[17,18] The demand for probiotics from natural sources as a remedy has been increasing day by day with the emergence of various diseases like diarrhea, and irritable bowel syndrome.[19] These bacteria grow and reduce gastrointestinal diseases by increasing the growth of beneficial pathogens and reducing the population of pathogens.[20] Firstly, LAB is safe and can be used in making dietary supplements. Secondly, these microorganisms are involved in fermentation and play an important role in making microflora of fermented foods.[21,22] LAB can also be used in food preservation by inhibiting microbial spoilage and shows an antagonistic effect against some food-borne pathogens by producing acetic acid, lactic acid, bacteriocin, carbon dioxide, and hydrogen peroxide.[23] LAB also shows some antimicrobial properties against pathogenic microorganisms.[24]

Multiple investigations on the cow milk have shown the titre of *Lactobacillus* as most abundant ranging from 1.0×10^2 to 3.2×10^4 CFU mL^{-1}. *Lactobacillus* is a gram-positive, rod-shaped, non-spore-forming, facultatively anaerobic, acid-tolerant bacteria.[25,26] *Lactobacillus* is generally recognized as a safe microorganism and hence can be used as a dietary supplement. These can be easily isolated from milk samples by culturing on De Man, Rogosa, and Sharpe (MRS) agar.[27] MRS agar contains some specific growth factors like sodium acetate, which suppress the growth of other bacteria and promotes the selective growth of *Lactobacillus* species.[6] *Lactobacillus* confers some specific biochemical properties like tolerance to pH, different temperature ranges, and the ability to ferment fructooligosaccharides, beta-galactosidase activity, antibiotic susceptibility, and helps to overcome problems like *Lactobacilli* are known to confer some health benefits to humans such as the increase in lactose intolerance beneficial effects on the intestinal flora, cure intestinal tract infections, stimulation of the immune system, reduction of inflammatory or allergic reaction; regulation of gut motility and promotion of a feeling of well-being.[28-30] *Lactobacillus* can remain active in the gastrointestinal tract and can also modulate the host's physiology, irrespective of its lifestyle. A number of common species of *Lactobacillus* are known to have beneficial effects.[31,32] The most abundant species of *Lactobacillus* found in cow milk are *L. acidophilus, L. plantarum,* and *L. casei*. In the present study, we isolated and characterized the *Lactobacilli* species from fresh raw milk from drawn from desi and crossbreed cows of Gurugram region, Haryana, India.

MATERIALS AND METHODS

Study design

The study was carried out at SGT University, Gurugram. The samples which are processed during the study were collected from the nearby area of the university. The duration of the study was from January to July i.e., 6-7 months.

Criteria for selection of cow for sample collection

The factors which are included in the study are:

- The cow is free from any disease and underlying medicated condition.
- The cow should not be on any kind of antibiotics.
- The cow is not supplied with dietary supplements.

The milk samples were collected from desi and cross-breed cows of local dairy farms in the Gurugram region of Haryana state, India. The number of samples taken was 12. Freshly drawn raw cow milk was collected in sterile air-tight 15 mL falcon tubes and transported using an icepack to the Department of Microbiology, Faculty of Allied Health Sciences, Shree Guru Gobind Singh Tricentenary University and Hospital, Gurugram. The collection tube was taken from the laboratory of SGT University, Gurugram. Pasteurized packaged cow milk sample was taken as a control sample for the experiment.

Isolation of *Lactobacillus*

The bacteria were isolated by preparing serial dilutions of the sample with sterilized normal saline; 0.1 mL of the diluted bacterial colony was pippetted and spread on MRS agar plate. Some of the samples were streaked on the MRS agar plates. The plates were then placed in the incubator at 37°C in anaerobic conditions for 24-48 hr.

Anaerobic conditions were created for the maximum growth of the bacteria using anaerobic jars.

After 48 hr, the bacterial colonies were then analyzed for morphological characteristics like cell shape, size, and texture on the selective media. Gram staining was done to determine whether the isolates are gram-positive or gram-negative. The biochemical tests include catalase, oxidase, citrate, indole, motility, and tolerance to some physiological conditions like growth at different temperature ranges, at different salinity, and at different pH. The suspected bacterial colonies were identified using Biomeuriex VITEK-2 ID GP Cards.

RESULTS

MRS Agar was used because it is selective media for the selective isolation of *Lactobacillus* and sometimes some other LABs also (Figure 1). It consists of sodium acetate, ammonium citrate, and polysorbate, which make it selective for the isolation of particular bacterial species. On incubation of diluted milk sample in MRS broth anaerobically for 24 hr, turbidity due to bacterial growth was observed. Table 1 and Figure 2 shows the morphological characteristics of the isolated colonies.

Table 1: Morphological characteristics of raw milk isolates.

ISOLATES	Colony Colour	Colony Shape	Colony texture	Cell Shape	Gram stain
Control	Off white	Circular, Elevated	Smooth	Rod	Positive
B1M1 (A)	white	Circular	Mucoid	Rod	Positive
B1M1 (B)	white	Branched, Elevated	Mucoid	Cocci	Negative
B1M2	Pale white	ND	Mucoid	Rod	Positive
B1M3	Pale white	ND	Mucoid	Cocci	Positive
B1M4	Off white	ND	Mucoid	Rod	Positive
B1M5	White	Circular, Elevated	Smooth, Creamy	Cocci	Positive
B2M1	White	Circular	Smooth	Rod	Positive
B2M2	Off white	ND	Mucoid	Rod	Positive
B2M3	White	Circular	Smooth	Rod	Positive
B2M4	Off white	ND	Mucoid	Rod	Positive
B2M5	white	Circular	Smooth	Rod	Positive
B2M6	Off white	ND	Mucoid	Rod	Position
B2M7	white	ND	Mucoid	Rod	Position

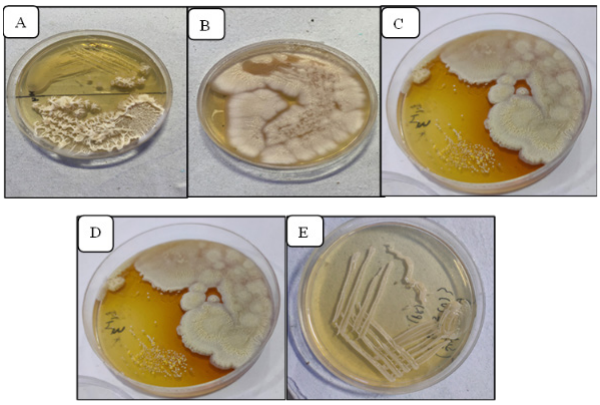


Figure 1: Growth of bacteria from raw milk samples of category-1 (cross-breed cow milk) on MRS Agar plates. (A) B1M1, (B) B1M2, (C) B1M3, (D) B1M4 and (E) B1M5.

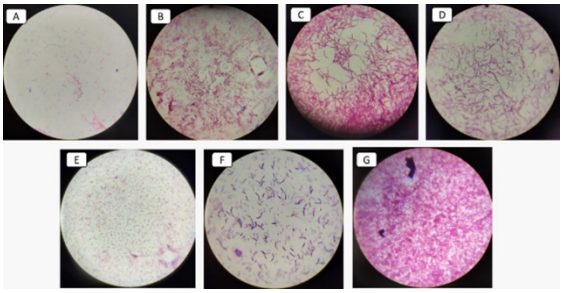
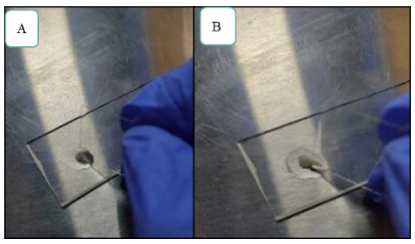
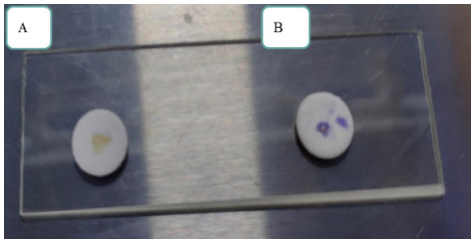


Figure 2: Gram stain and Microscopic view of raw cow milk samples of category-2 (Desi cows) (A) B2M1, (B) B2M2, (C) B2M3, (D) B2M4, (E) B2M5, (F) B2M6, (G) B2M7.

The results for biochemical characterization show that all of the bacterial isolates were negative for the Oxidase test, citrate test, sulfide indole motility test, and gelatinase test (Figure 3, Table 2). Four isolates were found which were catalase-negative and else were all catalase positive.



Catalase Test



Oxidase Test

Figure 3: Results of Catalase test and Oxidase test for raw milk isolates.

Table 2: Biochemical characterization of raw milk isolates.

Isolates	Catalase	Oxidase	Citrate	SIM	Gelatinase
Control	-	-	-	-	-
B1M1 (A)	+	-	-	-	-
B1M1 (B)	-	-	-	-	-
B1M2	+	-	-	-	-
B1M3	+	-	-	-	-
B1M4	+	-	-	-	-
B1M5	+	-	-	-	-
B2M1	-	-	-	-	-
B2M2	+	-	-	-	-
B2M3	+	-	-	-	-
B2M4	+	-	-	-	-
B2M5	-	-	-	-	-
B2M6	+	-	-	-	-
B2M7	+	-	-	-	-

Out of all the bacterial isolates, three bacterial colonies showed a resemblance with the characteristics shown by *Lactobacillus* according to Bergey's manual. Those bacterial colonies were isolated in this study. So, only those isolates were tested for tolerance under different physiological conditions and the isolates showed positive results under some conditions. The physiological conditions chosen were salinity, temperature ranges, and different pH (Table 3).

Table 3: Tolerance to Physiological Parameters.

Physiological Parameters	B2M1	B2M3	B2M5
Salinity			
2	++	+	++
4	++	+	+
6	+	+	+
8	+	+	+
pH			
4	-	-	-
5	-	+	-
6	++	++	+
7	+	+	+
Temperature			
25°C	+	++	+
37°C	++	++	++

Here, (++) shows very high growth, (+) shows moderate growth, and (-) shows no growth.

Vitek 2 ID Gp Card Result

The result for the identification of gram-positive cocci showed the presence of *S. epidermidis*. The Biomerieux identification system was able to identify the presence of suspected microorganisms with a 95 % probability in an analysis time of 5.82 hr.

DISCUSSION

Raw milk contains a wide variety of microflora because of the nutrients present in milk as studied in this study. A variety of bacteria, fungi, yeast, and molds can grow in milk because of its high nutritional value, a large amount of water, milk solids containing vitamins, minerals, carbohydrates, and a good amount of protein serve as good substrates for the growth of these microorganisms. Recent research has linked the emergence of new milk-derived bacterial pathogens like *S. aureus, E. coli, Pseudomonas* spp., *Bacillus,* and *Corynebacterium* spp. to increased public health and daily challenges.[33-35] Some regulations require proper milk handling and pasteurization to protect public health from milk-borne infections. However, such regulations are rarely followed in developing countries, raising the health risks associated with milk from developing countries and increasing the health risk of milk origin in developing countries. Likewise, we also got gram-positive cocci in our results which was confirmed by using the vitek identification system for gram-positive bacteria such as *S. epidermidis,* which gets contaminated in milk when the cow is suffering from mild mastitis, which cannot be seen by naked eyes and it may be contaminated from the skin of milk handler as *S. epidermidis* is a normal flora of epidermal layer of skin of humans. Some gram-positive rods which formed mucoid white with sticky colonies on MRS agar were also isolated but we were unable to confirm the microorganism in comparing the colony morphology with other papers it can be discussed that the bacteria may be *Listeria monocytogenes,* which is a food-borne pathogen found in milk, these bacteria also tend to form biofilms and resist the growth of other microorganisms. From all the bacteria isolated from raw cow milk, three isolates were tested for tolerance to physiological conditions. Those isolates were found to tolerate different temperatures, different salinity, and different pH. The properties shown by those isolates matched the characteristics shown by lactic acid bacteria from raw cow milk. Lactic acid bacteria are purple-colored rods that show positive results for gram staining; do not form spores, and non-motile bacteria that feed on carbohydrates. Lactose sugar from milk is commonly fermented by LABs into lactic acid, which imparts a distinct taste and odor to milk and milk products. *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Leuconostoc* spp., *Pediococcu*s spp., and *Bifidobacterium* spp. are examples of LABs. The most abundantly found

LAB is the *Lactobacillus* genera which form about 1.0×102-3.2×104 CFU mL^{-1} titer in milk. All other LABs are present in a lesser amount than *Lactobacillus*. Biochemical tests were done to test out the presence of certain enzymes and the ability of the bacteria to express those enzymes like catalase, oxidase, nitrate, sulfide, indole, motility, and gelatin hydrolysis. Some isolates gave a positive result for the tests while some were negative. The isolates which were catalase positive were having catalases while others lack catalase enzymes. Generally, species of the genus *Lactobacillus* are catalase-negative but sometimes *Lactobacillus plantarum* is known to give catalase-positive results. Because of their diverse metabolic profile and unique flavor-forming activity, LABs isolated from non-dairy sources have gained popularity in recent years. Plant-derived lactobacteria strains are more resistant to higher pH and salt concentrations, can ferment more types of carbohydrates, and have higher levels of stress tolerance than dairy-derived strains.

CONCLUSION

In this study, various bacteria were isolated on basal media as well as selective media for lactic acid bacteria. On blood agar, there were no bacterial isolates that showed characteristics like LABs. While on MRS agar three isolates were found which showed most of the properties and characteristics of LABs. Those isolates were gram-positive, rod-shaped bacteria which were suspected of *Lactobacillus* spp. Two of those isolates were catalase-negative and one was catalog-positive. All three isolates were oxidase negative, nitrate negative, and SIM negative, and also they showed negative results for the gelatin hydrolysis test. The isolates showed proper growth under different physiological conditions. The isolates showed growth at 5, 6, 7, and 8 pH but were unable to grow at pH 4. The tolerance of the bacterial isolates against different salt concentrations showed good growth at 2%, 4%, 6%, and 8% salinity. Also, the isolates were grown properly at high temperatures. These are all the properties shown by LABs also. The confirmation of the isolates is not yet done but these are suspected to be the common lactic acid bacteria.

FUTURE RECOMMENDATIONS

For further instance, new strategies should be implemented for creating anaerobic culture conditions. Also, for the identification of such bacteria molecular methods like gene sequencing and polymerase chain reaction (PCR) can be used efficiently for precise results. LABs after isolation can be implemented in food products to improve their nutritional value as well as their commercial cost. Many species of Lactobacillus can also be used in the food preservation process in food industries. LABs can also be used for adding aroma and flavour to varieties of food products as they produce a sour-sweet flavour on fermenting lactose into lactic acid. A few species of lactobacillus can also be employed in increasing the fertility of soil as Lactobacillus outnumbers hazardous bacteria in the food and water that animals ingest, and it improves their gut microbiota so that their first line of defense works more effectively. Lactobacillus works as a soil probiotic on the farm, making it an ideal medium for organic farming. It colonizes the soil and keeps diseases away.

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Authors received no funding for this study.

CONFLICT OF INTEREST

There is no conflict of interest among authors.

ABBREVIATIONS

LAB: Lactic Acid Bacteria; **CDC:** Centre for Disease Control and Prevention; **CFU:** Colony Forming Unit; **PCR:** Polymerase Chain Reaction.

SUMMARY

In this study, lactic acid bacteria were isolated from raw cow milk. Blood agar failed to show bacterial growth, however, MRS agar showed three isolates of Lactobacilli. Various biochemical tests were performed on the isolated bacteria at various pH range and salt concentrations. Molecular techniques are suggested reliable than conventional methods for Lactobacilli identification upto species level.

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