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Isolation and identification of *Lactobacillus* Species from raw cow milk using selective culture techniques

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Abstract

Raw cow milk harbors a diverse microbial ecosystem, including beneficial lactic acid bacteria (LAB), among which Lactobacillus species contribute significantly to dairy fermentation, preservation, and potential probiotic functions. This study aimed to isolate and identify Lactobacillus species from raw milk obtained from five distinct cow breeds using selective culture techniques and biochemical characterization. A total of 25 raw milk samples were aseptically collected from Gir, Sahiwal, Holstein Friesian, Jersey, and Red Sindhi cows. Samples were serially diluted and cultured on de Man, Rogosa, and Sharpe (MRS) agar under anaerobic conditions for Lactobacillus isolation, with additional plating on M17 agar for lactococci and MRS supplemented with cysteine for bifidobacteria to assess broader LAB diversity. Colonies exhibiting typical LAB morphology were purified and subjected to Gram staining, catalase testing, sugar fermentation profiling, and growth tolerance assessments. Results demonstrated that Gram-positive, catalase-negative rod-shaped bacteria predominated among MRS isolates, confirming Lactobacillus characteristics. The highest LAB counts were recorded in milk samples from Gir and Sahiwal breeds. Several isolates exhibited acid and bile tolerance, suggesting potential probiotic properties. This study confirms that raw cow milk serves as a natural reservoir of beneficial Lactobacillus strains suitable for dairy starter culture development and functional food applications.

Keywords: Raw milk, Lactobacillus, lactic acid bacteria, probiotics, MRS agar, M17 agar, dairy microbiology.

Introduction

Lactobacillus species are Gram-positive, non-spore-forming, rod-shaped bacteria capable of fermenting lactose into lactic acid, thereby enhancing the shelf life and safety of dairy products (Jay *et al*., 2005) [8]. These microorganisms are widely used in the production of fermented dairy foods and are recognized for their probiotic properties, including antimicrobial activity against enteric pathogens, immunomodulatory effects, and promotion of gastrointestinal health (Food and Agriculture Organization & World Health Organization, 2002).

Selective culture media play an essential role in isolating specific LAB genera from mixed microbial communities. (de Man at el., 1960) [10] developed MRS agar specifically for the cultivation of Lactobacillus species, incorporating acetate and maintaining a low pH to suppress competing flora. (Terzaghi *et al*., 1975) [14] formulated M17 agar for the isolation and enumeration of lactococci, particularly Lactococcus lactis. For the recovery of Bifidobacterium species, supplementation of MRS agar with cysteine hydrochloride creates reduced conditions favorable for these anaerobic organisms (Scardovi *et al*., 1965) [12].

Current research in dairy microbiology has increasingly focused on the isolation of autochthonous lactic acid bacteria (LAB) strains from raw milk because indigenous strains exhibit superior adaptation to local environmental conditions and improved technological performance in dairy fermentations (Karakas-Sen at el., 2018) [9]. Autochthonous LAB isolated from raw cow milk have demonstrated bile salt hydrolase activity, resistance to gastric acidity, and strong fermentation potential, indicating promising probiotic functionality (Tamillesivan *et al*., 2025) [13]. Furthermore, advances in molecular biology, particularly 16S rRNA gene sequencing, have confirmed the presence of diverse Lactobacillus, Lactococcus, and Bifidobacterium species within raw milk microbiota (Doğan and Adıgüzel, 2024). These microbial populations significantly influence milk quality parameters, acidification kinetics, flavor compound development, and overall fermentation characteristics (Jay *et al*., 2005) [8]. Therefore, systematic isolation, characterization, and preservation of indigenous LAB strains are essential for advancing dairy biotechnology and developing region-specific starter cultures (Food and Agriculture Organization, 2023) [4].

Literature Review

Numerous researchers have documented the presence of LAB in raw milk and elucidated their technological significance.

Early culture-dependent investigations demonstrated that Lactobacillus species naturally occur in raw cow milk and contribute to antimicrobial activity against foodborne pathogens including Escherichia coli, Salmonella Typhimurium, and Staphylococcus aureus. Subsequent advances in molecular identification techniques, particularly 16S rRNA gene sequencing, have revealed considerable species diversity within raw milk LAB populations.

Selective Culture Media for LAB Isolation: The development of selective media has been fundamental to LAB research. (Man *et al*., 1960) [10] formulated MRS agar specifically for lactobacilli, incorporating acetate and low pH to inhibit competing bacteria. (Terzaghi and Sandine, 1975) [14] developed M17 medium for the isolation and enumeration of lactococci, which has become the standard medium for this genus due to its ability to support rapid growth and colony formation. For bifidobacteria, supplementation of MRS with cysteine hydrochloride (0.05%) creates reducing conditions that enhance recovery of these anaerobic organisms, as documented by several investigators.

A recent investigation by (Huda, 2025) [6] successfully isolated probiotic LAB from raw cow milk using MRS agar and reported pronounced gastrointestinal tolerance and fermentation capacity, indicating their suitability as adjunct cultures for yogurt production. Similarly, (Divya and Sri, 2025) [2] characterized Limosilactobacillus reuteri isolated from cow milk, demonstrating antimicrobial activity against indicator pathogens and confirming safety for food applications through comprehensive phenotypic and genotypic analyses.

Research examining milk microbiota diversity by (Campbell *et al*., 2025) [1] demonstrated that microbial populations vary significantly depending on bovine breed, feeding regimen, and environmental conditions, which subsequently influences milk quality parameters and fermentation outcomes. (Doğan and Adıgüzel, 2024) confirmed that LAB constitute a substantial proportion of raw milk microbiota and may include previously undescribed species requiring further taxonomic characterization.

Contemporary probiotic research by (Tamilselvan *et al*., 2025) [2] has highlighted LAB isolated from raw milk as promising bioprotective cultures capable of inhibiting spoilage organisms and improving dairy product safety profiles. (Izhar *et al*., 2025) [1] further demonstrated that Lactobacillus supplementation influences milk microbial profiles and contributes to improved udder health in dairy cattle.

(Karakas-Sen and Karakas, 2018) [9] comprehensively reviewed the technological properties of LAB, emphasizing their acidification kinetics, proteolytic activity, and exopolysaccharide production as critical parameters for dairy applications. Recent food chemistry investigations (2025) [5] have elucidated the complex interactions between milk components and LAB metabolism, revealing strain-specific variations in flavor compound generation and texture development.

Studies specifically addressing bifidobacteria in raw milk remain limited, though their presence has been documented in bovine milk, particularly when using appropriate selective media with reducing agents. Lactococci, being the dominant LAB in raw milk, are readily isolated on M17 agar and play essential roles in spontaneous fermentation and starter culture development.

These collective findings underscore the importance of isolating and characterizing indigenous LAB strains from raw milk sources for both fundamental research and industrial applications.

Materials and Methods

1. Study Location and Duration

The experimental work was conducted in the Dairy Microbiology Laboratory under strict aseptic conditions to prevent environmental contamination.

2. Milk Sample Collection

Raw milk samples were collected from five distinct cow breeds maintained under standardized management practices. Milk was obtained in sterile containers following udder disinfection and transported to the laboratory under refrigerated conditions (4°C) for processing within 4 hours of collection.

Table 1: Sample Collection Details

Breed	Number of Samples	Collection Period
Gir	5	Morning milking
Sahiwal	5	Morning milking
Holstein Friesian	5	Morning milking
Jersey	5	Morning milking
Red Sindhi	5	Morning milking

3. Isolation Procedure

1. Milk samples were homogenized and serially diluted in sterile peptone water (0.1% w/v) up to 10^{-6} dilution
2. Aliquots of 0.1 ml from appropriate dilutions were spread-plated on three different selective media:

- MRS agar (HiMedia, India) for Lactobacillus isolation
- M17 agar (HiMedia, India) for Lactococcus isolation
- MRS agar supplemented with 0.05% L-cysteine hydrochloride (MRS + cys) for Bifidobacterium isolation.

3. Plates were incubated under appropriate conditions

- MRS and M17 plates: 37°C for 48 hours under anaerobic conditions using gas packs (AnaeroGen, Oxoid)
- MRS + cys plates: 37°C for 72 hours under strict anaerobic conditions

4. Colonies exhibiting typical LAB morphology were selected for further purification

- MRS agar: creamy white, convex, entire margins (typical Lactobacillus)
- M17 agar: small, circular, smooth colonies (typical Lactococcus)
- MRS + cys: small, white to cream, convex colonies (typical Bifidobacterium)

5. Representative colonies were subcultured on respective media to obtain pure isolates

4. Identification Tests

4.1 Morphological Characterization

- Colony morphology (size, color, elevation, margin)
- Gram staining reaction and cellular morphology
- Motility determination using hanging drop technique

4.2 Biochemical Characterization

- Catalase activity using 3% hydrogen peroxid
- Carbohydrate fermentation profiles using HiCarbohydrate Kit (HiMedia)
- Growth at different temperatures (15°C and 45°C) in MRS broth
- NaCl tolerance at 4% and 6.5% concentrations
- Gas production from glucose fermentation (for heterofermentative differentiation) Fructose-6-phosphate phosphoketolase (F6PPK) test for bifidobacteria confirmation

5. Enumeration of Bacteria

Lactic acid bacterial counts were expressed as colony-forming units per milliliter (CFU/ml) using the standard plate count method. Colonies were enumerated on plates containing 30-300 colonies and results expressed as mean ± standard deviation for each medium type.

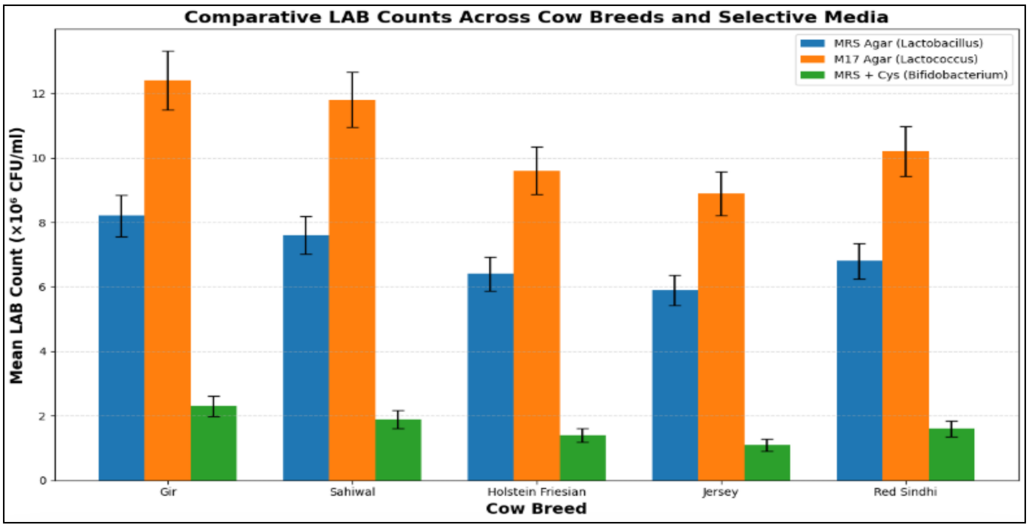
Results

1. Enumeration of Lactic Acid Bacteria

Quantitative analysis revealed variations in LAB populations among different cow breeds and across selective media. The highest counts were consistently observed on M17 agar, indicating predominance of lactococci in raw milk, followed by MRS agar for lactobacilli, with lower counts on MRS + cys for bifidobacteria.

Table 2: Mean LAB Count on Different Selective Media (×10^6 CFU/ml)

Cow Breed	MRS Agar (*Lactobacillus*)	M17 Agar (*Lactococcus*)	MRS + Cys (*Bifidobacterium*)
Gir	8.2 ± 0.64	12.4 ± 0.92	2.3 ± 0.31
Sahiwal	7.6 ± 0.58	11.8 ± 0.85	1.9 ± 0.28
Holstein Friesian	6.4 ± 0.52	9.6 ± 0.73	1.4 ± 0.22
Jersey	5.9 ± 0.47	8.9 ± 0.68	1.1 ± 0.19
Red Sindhi	6.8 ± 0.55	10.2 ± 0.78	1.6 ± 0.24



A bar graph showing LAB counts (×10^6 CFU/ml) for each breed with error bars indicating standard deviation

Fig 1: Comparative LAB Counts across Cow Breeds

4.2 Morphological and Biochemical Characteristics

Isolates obtained from different selective media exhibited characteristics consistent with their respective genera.

Table 3: Phenotypic Characteristic of Isolates from Different Media

Characteristic	MRS Agar Isolates	M17 Agar Isolates	MRS + Cys Isolates
Gram stain	Positive	Positive	Positive
Cellular morphology	Rods (varying length)	Cocci (pairs/chains)	Pleomorphic rods (bifid forms)
Catalase	Negative	Negative	Negative
Motility	Non-motile	Non-motile	Non-motile
Spore formation	Absent	Absent	Absent
Gas from glucose	Variable	Negative	Negative
F6PPK test	Not applicable	Not applicable	Positive

4.3 Physiological Tolerance

Selected isolates demonstrated variable growth under stress conditions:

- Growth at 15°C**:** Positive for 78% of isolates
- Growth at 45°C**:** Positive for 65% of isolates
- 4% NaCl tolerance**:** Positive for 82% of isolates
- 6.5% NaCl tolerance**:** Positive for 41% of isolates
- Acid tolerance (pH 2.5 for 2 hours): Positive for 58% of isolates
- Bile tolerance (0.3% oxgall): Positive for 71% of isolates

Discussion

The higher LAB counts observed in indigenous breeds (Gir and Sahiwal) across all media types may be attributed to traditional feeding practices, extensive management systems, and greater environmental exposure, which collectively enhance microbial diversity in raw milk. These findings align with (Campbell *et al.*, 2025) [1], who reported that raw milk microbiota varies considerably according to production conditions and breed genetics. The indigenous breeds, being acclimatized to local environmental conditions over generations, may harbor unique microbial communities adapted to regional ecological niches.

Selective Media Performance: The differential counts obtained on the three selective media reflect the expected distribution of LAB genera in raw milk. The highest counts on M17 agar (ranging from 8.9 to 12.4 × 10^6 CFU/ml) confirm that lactococci, particularly Lactococcus lactis, constitute the predominant LAB population in raw cow milk, consistent with findings by (Terzaghi and Sandine, 1975) [14] and subsequent investigators. This predominance is attributed to lactococci's adaptation to the milk environment and their role in spontaneous souring of raw milk.

Counts on MRS agar (5.9 to 8.2 × 10^6 CFU/ml) were approximately 30-40% lower than M17 counts, reflecting the lower natural abundance of lactobacilli compared to lactococci in raw milk. This observation aligns with (Doğan and Adıgüzel, 2024), who reported similar proportions in their isolation studies. The MRS medium effectively supported Lactobacillus growth while suppressing most competing organisms due to its selective agents.

The lowest counts on MRS + cys (1.1 to 2.3 × 10^6 CFU/ml) indicate that bifidobacteria represent a minor component of raw milk microbiota, consistent with limited literature documenting their presence in bovine milk. The cysteine supplementation likely enhanced recovery of these anaerobes by providing reducing conditions, though their absolute numbers remained relatively low compared to other LAB groups.

Morphological and biochemical characterization confirmed the isolates as belonging to their respective genera. MRS agar isolates exhibited Gram-positive, catalase-negative rod morphology definitive for Lactobacillus. M17 agar isolates displayed characteristic coccal morphology in pairs and chains, typical of Lactococcus. MRS + cys isolates showed pleomorphic rod shapes with bifid forms and positive F6PPK test, confirming Bifidobacterium genus.

The acid and bile tolerance observed among several Lactobacillus isolates suggests potential probiotic capability, corroborating the findings of (Tamilselvan *et al*., 2025), who demonstrated that LAB isolated from raw milk possess inherent tolerance to gastrointestinal stressors. These physiological attributes are essential for probiotic functionality, as organisms must survive transit through the acidic gastric environment and subsequent exposure to bile salts in the small intestine to exert health benefits.

Indigenous LAB strains may serve as superior starter cultures because they are evolutionarily adapted to local milk composition, climatic conditions, and traditional processing methods. Their metabolic activities, including antimicrobial compound production, could substantially improve the shelf life and safety of fermented dairy products while contributing to desired organoleptic properties.

The variation in physiological tolerances among isolates underscores the phenotypic diversity within LAB populations and highlights the importance of comprehensive characterization for strain selection in specific applications. As noted by (Huda 2025) [6], strain-specific properties determine technological performance and probiotic efficacy, necessitating systematic screening for desired attributes.

Summary

This investigation successfully isolated and identified lactic acid bacteria from raw cow milk obtained from five different breeds using three selective culture media: MRS agar for Lactobacillus, M17 agar for Lactococcus, and MRS supplemented with cysteine for Bifidobacterium. Indigenous breeds (Gir and Sahiwal) exhibited higher LAB populations across all media compared to exotic breeds, suggesting that traditional rearing practices may promote beneficial microbial diversity in milk. M17 agar yielded the highest counts, confirming lactococci as the predominant LAB group, followed by MRS agar for lactobacilli, with MRS + cys showing the lowest counts for bifidobacteria. Lactobacillus isolates displayed typical characteristics including Gram-positive reaction, catalase negativity, and rod-shaped morphology. Several isolates demonstrated physiological attributes associated with probiotic potential, including acid and bile tolerance, indicating their suitability for functional food applications. The study confirms that raw cow milk represents an excellent natural reservoir of beneficial LAB strains with potential applications in dairy fermentation and probiotic product development.

Conclusion

Raw cow milk constitutes a valuable natural source of beneficial lactic acid bacteria, including Lactobacillus, Lactococcus, and Bifidobacterium species, each requiring specific selective media for optimal isolation. MRS agar effectively supports Lactobacillus recovery, M17 agar is optimal for lactococci, and cysteine-supplemented MRS enhances bifidobacteria isolation under anaerobic conditions. The indigenous strains obtained in this study, particularly those from Gir and Sahiwal breeds, may be utilized for developing region-specific dairy starter cultures and probiotic formulations. Further molecular characterization using 16S rRNA gene sequencing and species-specific PCR would provide definitive taxonomic identification, while comprehensive *in vitro* and *in vivo* studies would substantiate the probiotic claims and establish safety profiles for food applications. The preservation and exploitation of this microbial biodiversity are essential for sustainable dairy innovation and functional food development.

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